CHARACTERIZATION OF ANTIBACTERIAL ACTIVITY OF A NOVEL ENVIRONMENTAL ISOLATE OF *SERRATIA PLYMUTHICA*

By

Mai Mohammed AL-Ghanem

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Edinburgh

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ABSTRACT

Serratia species are an important source of antimicrobial compounds with numerous studies concerning their production of secondary metabolites with applications in medicine, pharmaceutics and biotechnology. This study continued investigations on the antibacterial activity of an environmental isolate of *Serratia plymuthica.* This activity could be detected on agar plates against Gram-positive bacteria.

A previous study reported the isolation of Km^R transposon mutants deficient in the activity identified on agar plates but appeared to possess another activity secreted in liquid media. Those mutants had Tn*5* insertions into genes encoding polyketide synthases (PKSs). The multiple antibacterial activities of *S. plymuthica* indicate the production of antimicrobials that can diffuse into aqueous environments in addition to the ones closely bound to the outer cell surface and secreted into solid mediums. This raised the question about the relationship between the two activities, were they distinct or related?

This study investigated the secreted antibacterial activity of *S. plymuthica* using transposon mutagenesis to create mutants deficient in the secreted antibacterial activity and to identify the genes involved in antibacterial production. Cultivation parameters such as; culture medium, aeration and temperature had a clear impact on the production of the secreted antibacterial activity. Growing *S. plymuthica* in MRS broth at 16^{o}C for 48h-72h produced the highest amount of inhibitory activity detected in the CFCS. The secreted antibacterial activity was thermo-stable and remained active even after sterilization (121°C/15mins) and was insensitive to methanol, Proteinase K, Tween and pH. However, the antibacterial activity faded after few days even when stored at (4^{o}C). The antibacterial activity was bactericidal and targeted a sub-set of Gram-postive bacteria especially *Bacillaceae*. The CFCS precipitated in the presences of solvents such as methanol, Tris-buffer and Tween. Structural characterization of the antibacterial compound(s) was confirmed via NMR and COSY spectrometry and showed the presence of two polyketide compounds resembling the macrolide antibiotics erythromycin and rapamycin produced by type I PKSs. Erythromycin and rapamycin are anti-bacterial and immune-suppressant compounds.

Results of this study indicate that the secreted antibacterial activity of *S. plymuthica* is synthesized by PKSs that are distinct from other known *Serratia* PKSs. Results are also consistent with the possibility of *S. plymuthica* possessing separate biochemical pathways leading to two distinct antibacterial activities or a branched biochemical pathway. However, future studies are needed to reveal if the genes responsible for the two activities are in

separate genomic locations and regulated in different manners to prove that the strain possesses two separate biochemical pathways leading to the two distinct activities. Further work is needed to unlock these unanswered questions. This includes genomic studies in order to identify new secondary metabolites with future importance that might be produced by this novel strain of *S. plymuthica*.

DEDICATION

In the name of Allah, the most merciful

The Holy Quran says in chapter No. 9, At-tawbah, verse No. 105: "And Say, do as you will, for Allah will see your deeds, and so will his messenger and the believers''

This thesis is dedicated to

My dear father

To whom Allah bestowed with esteem and dignity, he who taught me bestowal without delay, to whom I bear his name with proud, I pray to the almighty God to prolong your age so that you can see the fruit which is about to ripe after a long patience. Your words will remain as the stars that guide me today, tomorrow and forever.

My dear mother

My inspiration in life, to the meaning of love, affection and devotion, to the smile of life and secrete of existence, to whom the prayer of her was the secrete of my victory and affection was the cure of my grievances.

My parents. How can anyone thank the sun for enlighting the world?

God bless you.

ACKNOWLEDGEMENTS

In such moments, the pen stops to think for a while before drawing the letters that form the words. The words scatter and try in trifle to be gathered as lines.

All the appreciation and thanks are not enough for my beloved country (Qatar), which was my support in my long journey to seek knowledge.

The thanks and appreciations are also extended to His Highness (Sheikh Tamim Bin Hamad Al Thani), may the almighty God protect him for his caring for knowledge and scholars, so that Qatar would be the brightening light of the Gulf and continues to hold the flag of knowledge and progress.

May Qatar remains proud and bounteous.

May Qatar remains a brightening light.

DECLARATION

I, Mai Mohammed Al-Ghanem, hereby declare that I am the author of this thesis. All the work described in this thesis is my own, except where stated in the text. Results presented in this work have not been previously used in an application for a higher degree. All sources of information are acknowledging by means of references.

Mai Al-Ghanem

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ABBREVIATIONS

16S rRNA	16S ribosomal RNA
g	Gravitational acceleration
AS	Activated sludge
AHL	N-Acyl homoserine lactone
Amp	Ampicillin
β-lactam antibiotics	Beta-lactam antibiotics
B. subtilis	*Bacillus subtilis*
F	*Bacillus cereus* variant of *mycoides*
B. cereus	*Bacillus cereus*
bp	Base pair
BHI	Brain Heart Infusion Agar
$CaCl_2$	Calcium chloride
C. albicans	*Candida albicans*
C. tropicalis	*Candida tropicalis*
C. krusei	*Candida krusei*
COSY	Correlation spectroscopy
CFCS	cell-free culture supernatant
CCS	Concentrated culture supernatant

DEBS	6-deoxyerythronolide B synthase
DNA	Deoxyribonucleic acid
DMSO-*d6*	Dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EB	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
HPLC-MS	Liquid chromatography–mass spectrometry
h	Hours
HIV	Human immunodeficiency virus
Inh	Transposition inhibitor
IE	Inside end
Km	Kanamycin
Km^R	Kanamycin resistant
K. pneumoniae	*Klebsiella pneumoniae*
KCl	Potassium Chloride

Kb	kilobase
LB	Luria Bertani Agar
MRSA	Methicillin-resistant *Staphylococcus aureus*
mRNA	Messenger RNA
ml	Milliliter
µl	Microlitre
µg	Microgramme
mins	Minutes
M	Molar solution
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MRD	Maximum recovery diluent
MRS	De Man, Rogosa and Sharpe
MWCO	Molecular weight cut-off
mbar	Millibars
NA	Nutrient Agar
NB	Nutrient Broth
NaCl	Sodium Chloride

NRPSs	Non-ribosomal peptide synthetases
NPs	Natural products
N	Number of times
NMR	Nuclear magnetic resonance
OD_{600}	Optical density at 600 nm
OE	Outside end
oriT	Origin of transfer
Pig	Prodigiosin
MOPS	Propanesulfonic acid
P. aeruginosa	*Pseudomonas aeruginosa*
ppm	Parts per million
PKSs	Polyketide synthases
PKS	Polyketide synthase
PCR	Polymerase chain reaction
pH	Power of hydrogen
QS	Quorum sensing
RT	Room temperature
RbCl	Rubidium chloride

rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S	*Salmonella enterica* variant of *enteritidis*
sec	Second
SD	Standard deviation
SOC buffer	Super optimal broth with catabolite repression
SlyA	Transcriptional regulator of *Salmonella typhimurium*
S. aureus	*Staphylococcus aureus*
S. plymuthica	*Serratia plymuthica*
S. marcescens	*Serratia marcescens*
S. enterica var. Enteritidis	*Salmonella enterica var enteritidis*
S. cerevisiae	*Saccharomyces cerevisiae*
S. diastaticus	*Saccharomyces diastaticus*
tRNA	Transfer RNA
Tnp	Transposase
TV	Total volume
TAE	Tris Acetate-EDTA buffer
TFB2	Transformation buffer

VOCs	Volatile organic compounds
V	Volt
VRSA	Vancomycin resistant *Staphylococcus aureus*
WHO	World Health Organization
YPD	Yeast Extract-Peptone-Dextrose Agar

CHAPTER 1: INTRODUCTION

1.1 Microbial natural metabolites

For many years now, microorganisms have been a fascinating area of study. Researchers have realized the importance of these tiny microbes for their ability to produce various useful natural metabolites and compounds with uncultured bacterial species being a rich source of bioactive molecules (Genilloud, 2014; I´oca *et al*., 2014). Microbial secondary metabolites are low molecular weight substances produced at later stages of microbial growth. Production can also be triggered by extrinsic stimuli and the availability of nutrients like nitrogen, phosphorous and carbon (Ruiz *et al*., 2010; Masschelein *et al*., 2015a). Natural metabolites represent an enormous group of organic compounds that do not play essential roles in the normal growth and development of the producing microorganisms but merely confer a selective advantage in complex microbial communities as a biological defense mechanism against predators (Lorente *et al*., 2014; Hellberg *et al*., 2015; Hibbing *et al*., 2010). The biosynthesis of bacterial secondary metabolites involves multiple enzymatic pathways and reactions brought about by structural diversity which contribute to their highly specific mechanisms of action (Cragg & Newman, 2013). Secondary metabolites include isoprenes, oligosaccharides, peptides, polyketides, β-lactam rings and aromatic compounds (Masschelein *et al*., 2013; Matilla *et al*., 2016b; Müllera & Wink, 2014).

The majority of antibiotics are derived from bacterial metabolites and soil bacteria including; *Streptomyces*, *Actinomycetes* and *Mycobacteriaceae* which are the main source of novel secondary metabolites with more than 20,000 products having been characterized including anti-helmintic, immuno-suppresant, cytotoxic, anti-inflammatory, anti-malaria and antiviral compounds (Gerc *et al*., 2012; Abel *et al*., 1999; Peláez, 2006). *Bacillus* species are major producers of antimicrobials, such as bacteriocin. The bacteriocin of *B. licheniformis* holds potent activity against Gram-positive bacteria (Manivasagan *et al*., 2014; Matilla *et al*., 2015). *Streptomyces* bioactive compounds include dithiolopyrrolones and Bleomycin (Trautman & Crawford, 2016; Sanchez *et al*., 2010 b). Research revealed the production of the antibiotic Actinorhodin by *Streptomyces coelicolor* which exhibit potent activity against Gram-positive bacteria (Rahman *et al*., 2010; Katz & Baltz, 2016; Mak *et al*., 2014). The Gram-negative bacteria *Pantoea agglomerans* produces the pseudo-peptide antibiotic Andrimid which displays broad spectrum antibacterial activities and anti-cancer

properties. *Mycobacteriaceae* produce the antibiotic elansolid with inhibitory activity against Methicillin-resistant *S. aureus* (Müllera & Wink, 2014; Pidot *et al*., 2014). *Aspergillus nidulans* produces the anti-tumour compound terrequinone A while, thiolactomycin isolated from *Nocardiaha* shows anti-malarial and antibacterial activity (Yurkovich *et al*., 2017; Peláez, 2006).

1.2 History of antibiotics

The history of antibiotics began in 1928 when Alexander Fleming observed that the growth of a plate of *Staphylococcus aureus* had been inhibited by a mold contamination. This mold was later identified as *Penicillin notatum* (Kümmerer, 2009; Saleem & Iqbal, 2015). This discovery was one of the most influential moments in human kind history and in the following years; penicillin was used as the first weapon to combat the most common infectious diseases in that era like pneumonia, gonorrhea and rheumatic fever. The so called ‘‘golden age’’ of antibiotics was mainly from the 1940s to the 1960s during which most of the major classes of antibiotics were produced. These include β-lactams, aminoglycosides, tetracyclines, macrolides and chloramphenicol (Wencewicz, 2016; Masschelein *et al*., 2015a). The majority of current drugs are based on the chemical modification of existing antibiotics with only few being fully synthetic like fluoroquinolones. An antibiotic can be defined as a bio-chemical compound produced by a microbe which kills or inhibits the growth of other microorganisms in low concentrations (Challinor & Bode, 2015; Davies & Davies, 2010; Kasanah & Hamann, 2004). No one can deny the importance of antibiotics, they are used to treat a wide range of bacterial infections and used as antitumor and anti-parasitic agents. They are extremely essential components of modern medicine, especially in intensive care, organ transplantation, cancer chemotherapy and surgical procedures. Antibiotics are also used in agriculture, aquaculture and in food processing (Marti *et al*., 2014; Meek *et al*., 2015; Pires *et al*., 2017).

1.3 Antibiotic Classification

Antibiotic classification is based on various chemical properties. These include, mechanism of action, cellular components they affect and whether they induce cell death (bactericidal drugs) or merely inhibit cell growth (bacteriostatic drugs) (Kohanski *et al*., 2010; Martinez, 2014; Dwyer, 2015). Research revealed that in reality pure classes of antimicrobial compounds (bactericidal/ bacteriostatic) do not exist and if so called "bactericidal" agents fail to kill every microorganism within a defined period then these agents will be called bacteriostatic (Pankey& Sabath, 2004; Rodloff *et al*., 2008). The most important cellular targets of antibiotics are shown in Figure 1.1 and include DNA replication, cell wall synthesis, protein synthesis and metabolic functions (Procópio *et al*., 2012; Lewis, 2013).

1.3.1 Protein synthesis inhibitors

1.3.1.1 Aminoglycosides and Tetracyclines

Aminoglycosides are one of the oldest groups of antibiotics, with streptomycin being the first member to be discovered in 1944. Aminoglycosides are hydrophilic compounds and consist of amino-sugars connected through glycosidic bonds. Examples include kanamycin, gentamicin and streptomycin (Hoerr *et al*., 2016; Procópio *et al*., 2012). This group of antibiotics is still used today in clinical practices despite their nephro-toxic side effects. They are bactericidal with broad-spectrum activity against aerobic Gram-positive and Gram-negative bacteria (Chellat *et al*., 2016). They target the 16S rRNA of the 30S–50S ribosome junction and disturb the stability of t-RNA binding to ribosomes which leads to the misreading of genetic code and the incorporation of incorrect amino acids into peptide chains. These abnormal proteins affect the bacterial cell membrane permeability leading to an increase in aminoglycoside uptake (Fourmy *et al*., 1996; Fair & Tor, 2014). Resistance occurs through modification of the target ribosome by methylation and by the action of enzymes like aminoglycoside N-acetyltransferases and efflux pumps (Mak *et al*., 2014; Katz & Baltz, 2016). Tetracyclines are bacteriostatic broad-spectrum antibiotics. In 1945, chlortetracycline was the first drug to be discovered within this group but, it was not until 1948 that it was used clinically (Katz & Baltz, 2016). Other examples include, oxytetracycline,

clomocycline and methacycline. The chemical structure of this class consists of an octa-hydro-tetracene skeleton. This group is active against Gram-positive and Gram-negative bacteria, Vancomycin-resistant *Enterococcus*, *Clostridium difficile* and protozoan parasites. Tetracyclines display only minor side effects which make them favorable in treating human bacterial infections (Procópio *et al*., 2012; Chellat *et al*., 2016). Tetracyclines act by penetrating the outer bacterial cell membrane into the cytoplasm, affecting the metal-ion concentration and causing an increase in pH. This creates a metal-ion/tetracycline complex which inhibits the binding of the aminoacyl tRNA to its designated ribosomal site. Resistance occurs via ribosomal modification induced by the *tet* and *otr* genes, inactivating enzymes and efflux pumps (Nguyen *et al*., 2014; Chopra & Roberts, 2001).

1.3.1.2 Chloramphenicol, Macrolides and Lincosamides

Chloramphenicol is a bacteriostatic antibiotic with broad spectrum activity. This class of antibiotics act on the 50S ribosomal subunit and prevent amino acid transfer by inhibiting peptidyl transferase and protein synthesis (Rahal *et al*., 1979).

As chloramphenicol, macrolides also act on the 50S ribosomal subunit and inhibit the synthesis of peptide chains. Macrolides are macro-cyclic lactones with deoxy-sugars. The first macrolide to be discovered was Erythromycin in 1949 isolated from *Actinobacteria* (Saleem & Iqbal, 2015). Others include tylosin and spiramycin. Macrolides display broad-spectrum activity against Gram-positive and Gram-negative bacteria. Macrolides are bacteriostatic but display bactericidal activity against *Streptococcus pneumoniae* and *Haemophilus* influenzae. Resistance occurs via Mono-methylation of rRNA (Katz & Baltz, 2016; Mak *et al*., 2014).

Lincosamides include Lincomycin, Clindamycin and Pirlimycin. These antibiotics inhibit protein synthesis by binding to the 50S ribosomal subunit with the inhibition of peptidyl transferases. Lincosamides are effective against anaerobic species and Gram-positive bacteria and considered both bactericidal and bacteriostatic (Leclercq, 2002; Fair & Tor, 2014).

1.3.2 Cell Wall Synthesis Inhibitors

1.3.2.1 Glyco-peptides and Beta-lactam antibiotics

Glyco-peptides and Lipo-glyco-peptides antibiotics include Vancomycin, Teicoplanin, and Avoparcin. Glyco-peptides are known as "Last resort antibiotics" in human medicine due to their highly toxic side effects. Glyco-peptides are bactericidal with narrow spectrum activity but effective against Gram-positive bacteria. Glyco-peptides interfere with penicillin-binding protein enzymes like transpeptidases and inhibit transglycosylation and transpeptidation which contribute to cell wall synthesis leading to bacterial cell death. Resistance occurs when the C-terminal D-alanyl-D-alanine of lipid II replaces the D-alanyl-D-lactate which in turn decreases the antibiotic affinity to interact to its cell wall target (Marcone *et al*., 2010; Chellat *et al*., 2016). Regarding Beta-lactam antibiotics, they are a group of compounds with diverse molecular structure but share a common four-membered β-lactam ring. The first Beta-lactam antibiotic to be discovered was benzyl-penicillin (Penicillin G). Other examples include, methicillin, ampicillin and amoxicillin (Saleem & Iqbal, 2015; Dantas & Sommer, 2014). They are bactericidal with low toxic side effects and broad-spectrum activity against various bacterial infections and diseases like anthrax, tetanus, streptococcal infections, UTI and enteritis (Kong *et al*., 2009; Katz & Baltz, 2016).

β-lactam antibiotics are inhibitors of cell wall synthesis. This occurs when the beta-lactam ring structure binds to penicillin-binding proteins located in the cell membrane which leads to autolysis and bacterial cell death (Fair & Tor, 2014). Resistance mechanisms involve the modification of cellular membrane channels and preventing the antibiotic from binding to penicillin-binding proteins also efflux pumps that eject the antibiotic out of the cell and antibiotic inactivation via hydrolysis of the β-lactam ring mediated by β-lactamase enzymes (Kohanski *et al*., 2010).

1.3.3 Nucleic acid synthesis inhibitors

1.3.3.1 Fluoroquinolones, Rifamycins and Quinolones

Fluoroquinolones and Rifamycins are bactericidal antibiotics that inhibit nucleic acid synthesis by binding to DNA gyrase and topoisomerase IV leading to a defect in DNA and cell death. Fluoroquinolones include, Enrofloxacin and Ciprofloxacin. Rifamycins include Rifampin, and Rifabutin. Resistance is due to mutations in topoisomerase IV and DNA gyrase (Hooper, 2001; Zhanel *et al*., 1999).

Quinolones are bactericidal antibiotics with broad-spectrum activity against Gram-positive and Gram-negative bacteria. These antibiotics are used in the treatment of skin, lung and urinary tract infections. This group of antibiotics target DNA replication. The topology of DNA is controlled by DNA Gyrase which has an important function in DNA and mRNA synthesis (Dantas & Sommer, 2014; Saleem & Iqbal, 2015). Quinolones create a complex with topoisomerase and prevent DNA replication and affect nucleic acid metabolism leading to cell death. Resistance occurs through target modification by mutations of the genes *gyrA* and *parC* (Pankey & Sabath, 2004; Kohanski *et al*., 2010).

1.3.4 Inhibitors of metabolic functions

1.3.4.1 Sulfonamides and Diaminopyrimidines

Sulfonamides and Diaminopyrimidines are bacteriostatic antibiotics that inhibit folic acid synthesis by interfering with the function of the enzyme dihydropteroate synthetase from adding paraaminobenzoic acid to the structure of folic acid. Sulfonamides include sulfadiazine and sulfamethoxazole (Kolaczek *et al*., 2014; Huovienen, 1987). Both groups display broad-spectrum activity against Gram-positive and Gram-negative bacteria and protozoal parasites. Sulfonamides resistance has been recorded in Gram-negative bacteria like some strains of *Escherichia coli* that contain plasmid encoded resistance genes namely *sul1*, *sul2*, and *sul3* (Arabi *et al*., 2015; Huovinen *et al*., 1995).

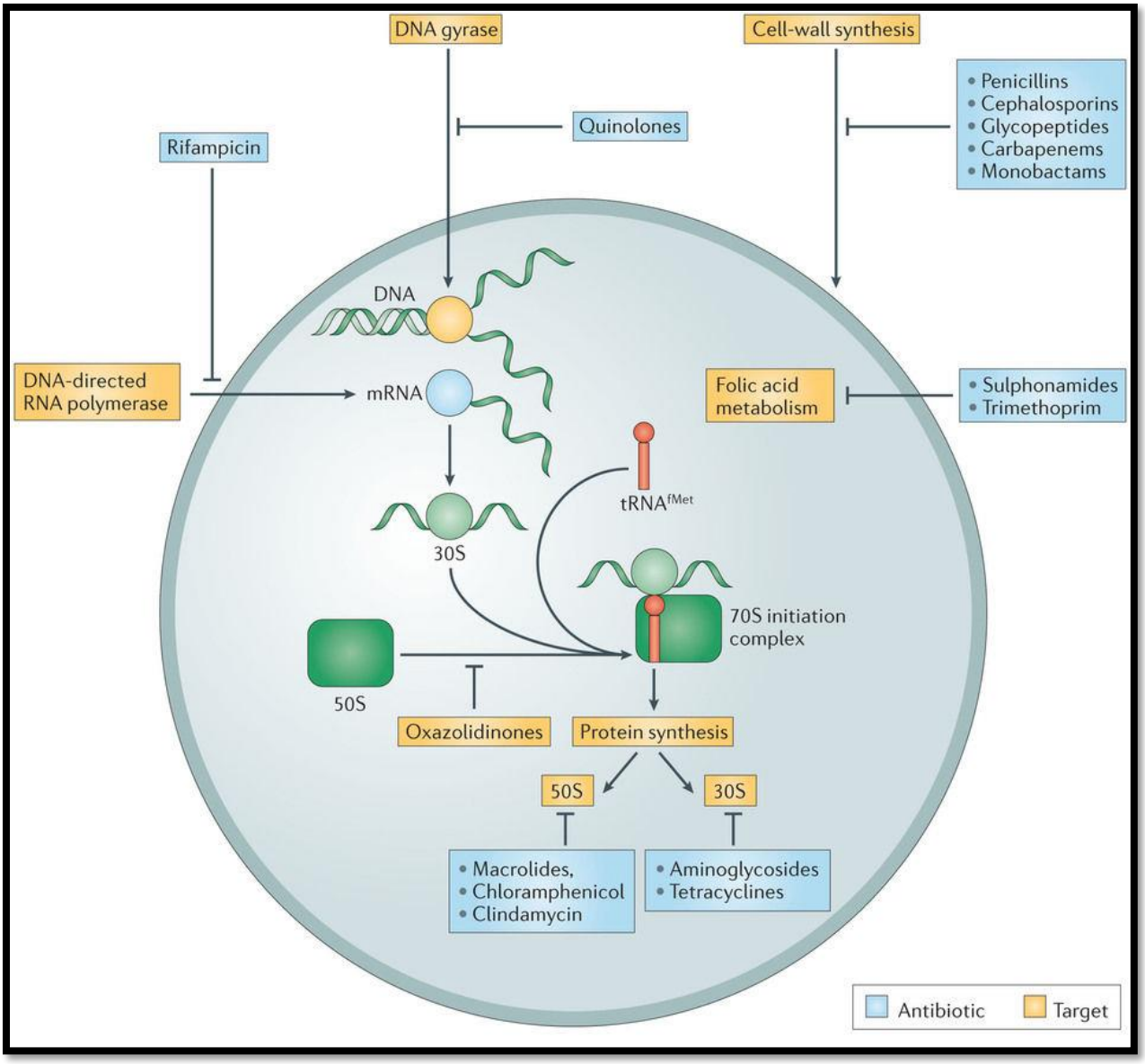

Figure 1.1: Main bacterial cellular targets of antibiotics (Lewis, 2013).

1.4 Antibiotic resistance

Infectious diseases are the second leading cause of death killing 3 million people each year around the world. The rapid development of antibiotic resistance is one of the biggest medical threats of modern times. The emergence of community and hospital-acquired infections of resistant pathogens (Figure 1.2) imposes serious threats in community and hospital settings (Navarri *et al*., 2016; Spížek *et al*., 2010; Sanchez *et al*., 2010a). Examples include methicillin and vancomycin resistant *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis* (Barrett & Barrett, 2003; Dancer, 2008). Infections caused by penicillin resistant *Streptococcus pneumoniae* is a public health concern in Qatar causing community-acquired pneumonia, bacteremia, meningitis and otitis and mostly affecting the elderly and young children (Elshafie & TajAldeen, 2016).

Gram-negative opportunistic pathogens like *E. coli* and *P. aeruginosa* are extremely worrying since they are the cause of common bacterial infections affecting human health with these strains being highly resistant to antibiotics (Hoerr *et al*., 2016). In recent years, the prevalence of untreatable gonorrhea infections become of great concern due to the emergence of a new strain of *Neisseria gonorrhoeae* that is extremely resistant not only to the first-line treatment of antibiotics but to extended-spectrum cephalosporins (Unemo & Nicholas, 2012).

More than 70% of bacterial pathogens causing infectious diseases have become resistant to at least one of the drugs used in the treatment of these infections. Resistance has rapidly developed from resistance to single classes of antibiotics to multidrug resistance and extreme drug resistance (Davies & Davies, 2010). Examples include multi-drug resistant *Clostridium difficile* and the rise of hospital acquired *Acinetobacter baumanii* infections affecting mainly immune-compromised patients in intensive care units (Marti *et al*., 2014).

In 2013, the World Health Organization WHO reported more than 400,000 new cases of multidrug-resistant tuberculosis as well extensively drug-resistant tuberculosis. Antibiotic resistance is a major challenge to treat infectious diseases, not to mention the enormous clinical and financial burden on health care systems (Blair *et al*., 2015). Patients affected by infections caused by drug-resistant bacteria are at risk of serious health problems and high consumption of medical resources (Fischbach & Walsh, 2009). Fortunately, according to the European Antimicrobial Resistance Surveillance Network EARS-Net, in recent years a dramatic change in

some of the resistant strains has been noticed in which improved hospital practices, such as cleaning and disinfection of surfaces, resulted in a decrease of transmission of MRSA and VRE between patients, emphasizing the importance of good housekeeping in healthcare facilities to reduce the risk of infections. However, there have been reports of increased resistance in other bacterial strains like *Acinetobacter* with notable high mortality rates (Wang *et al*., 2013; Fair & Tor, 2014; Lee *et al*., 2013).

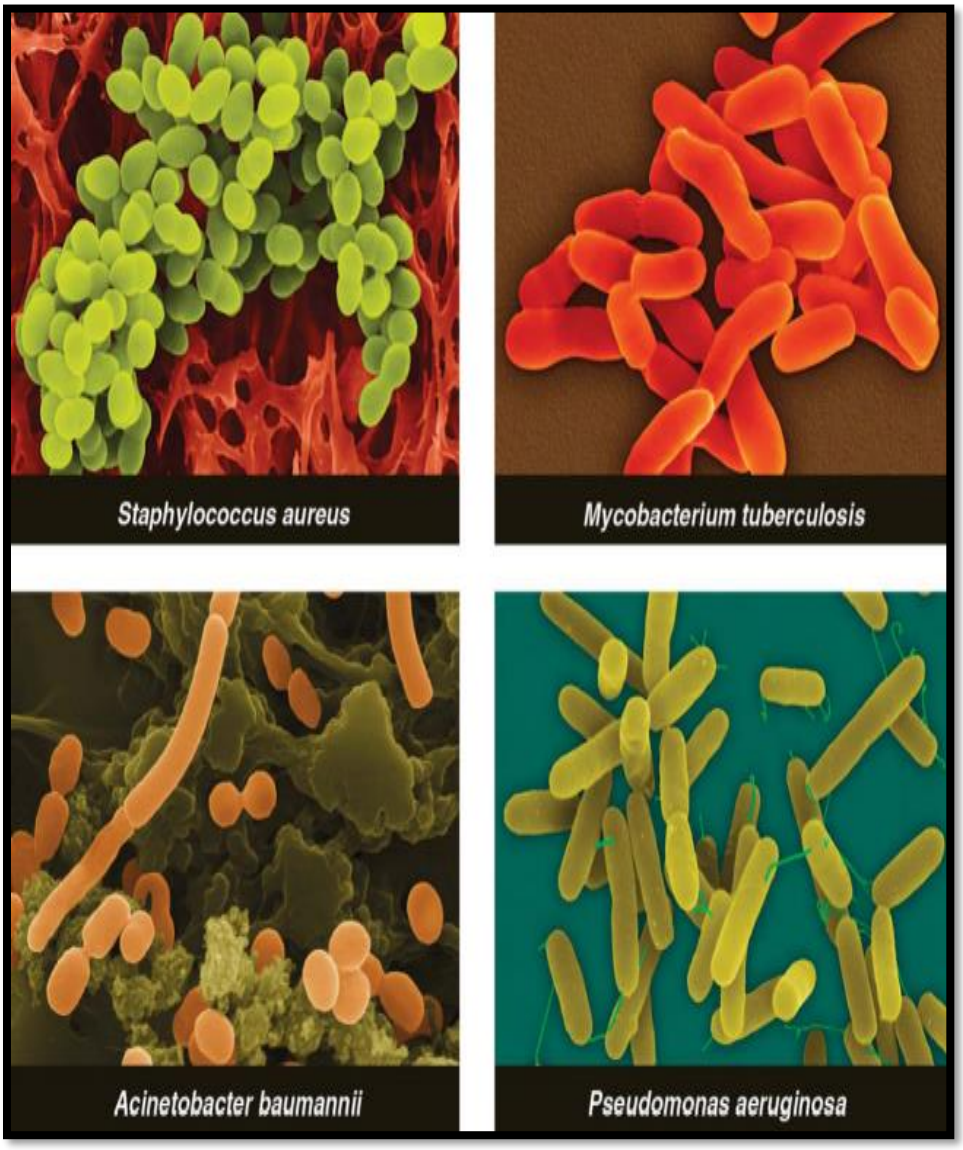

Figure 1.2: Examples of the most common resistant bacterial strains causing community and acquired infectious disease (Fischbach & Walsh, 2009).

1.4.1 Origin of antibiotic resistance

The emergence and spread of antibiotic resistance and antibiotic resistance genes is a major modern dilemma affecting various countries around the world. A lack of awareness of antimicrobial resistance among health care workers and patients allow this dilemma to propagate. Effective antimicrobial prescribing is a challenge as well as creating a balance between the dosage, duration of treatment and severity of the disease. More than 90% of antibiotics in Europe are prescribed to non-hospitalized patients, 46% in Brazil and 100 % in Nigeria and Sudan. In Qatar, 35 % of prescribed medications from private clinics were for antibiotics. Access to nonprescribed antibiotics also include purchases over the counter and online market companies (Marti *et al*., 2014; Davies & Davies, 2010; Keown *et al*., 2014).

Inadequate investments in the research of novel antibiotics and treatments as well as inappropriate practices (Figure 1.3) all contributed to selection pressure and the spread of resistant genes into human pathogens as demonstrated by the presence of the same resistant genes of soil bacteria in human pathogens. Examples include, patient self-medication, non-compliance with prescribed dosages (Bell *et al*., 2014; Black *et al*., 2014).

Some studies claim that levels of antimicrobial resistance are proportional to antibiotic consumption with observed resistance to penicillin and streptomycin shortly after their introduction in clinical practices. The release of antibiotics such as vancomycin, and the bio-chemical changes in natural ecosystems, altered the biological dynamics of microorganisms and lead to the selection of resistance (Elshafie &TajAldeen, 2016; Allen et al., 2014; Martinez & Baquero, 2014).

The dissemination of resistant bacteria had major effects on animal husbandry and even minute amounts of antibiotics can inflict a selective pressure on clinical bacterial strains. In Europe, it is prohibited to use antibiotics as growth promoters, although some like streptomycins are still used in crop growing and bee-keeping. Current sensitive bacterial pathogens can predominate in bacterial communities even if the designated antibiotic is absent emphasizing the absence of reversibility in clinical settings (Bell *et al*., 2014; Kümmerer, 2009; Saleem & Iqbal, 2014).

Poor sanitary conditions support the growth of pathogenic bacteria and the spread of antibiotic-resistant pathogens and their dissemination through water bodies which might reach farms and

wild animals (Wang *et al*., 2013; Yang *et al*., 2014). Antibiotic-resistant bacteria have been cultured from numerous rivers around the world which originated from water treatment plants and sewage systems. Water samples isolated from New Delhi in India, revealed the presence of bacterial antibiotic resistance strains as a result of contamination of water supplies by human faeces; emphasizing the need for appropriate treatment systems to prevent the spread of antibiotic resistance. Antibiotic resistance microorganisms and antibiotic resistance genes have been detected in seawater, beach sand, sediments and drinking water (Moskot *et al*., 2012; Marti *et al*., 2014; Tello *et al*., 2012).

Selection of resistant bacteria can even occur at low antibiotic concentrations similar to those found in some aquatic environments which can lead to antibiotic resistance. Marine bacteria isolated from the Baltic Sea have been found to be resistant to several antibiotics including kanamycin, ampicillin, chloramphenicol and tetracycline (Meek *et al*., 2015). Persistent selective pressure from antibiotic residues in wastewater and the presence of various species of microorganisms in activated sludge (AS) contribute to horizontal gene transfer of antibiotic resistant genes between different microbial communities mediated by mobile genetic elements such as plasmids and transposons. Antibiotic resistant bacterial strains derived from landfill disposal of sludge can disseminate into ground water and soil and remain in those environments even in the absence of antibiotic selection. Bacterial pathogens such as *Aeromonas* have been detected in sewage treatment with tetracycline and aminoglycoside resistance genes (Yang *et al*., 2014; Marti *et al*., 2014).

1.4.2 The resistome

Antibiotic resistance is a natural phenomenon and can exist even in the absence of antibiotics. Studies suggest that the resistome is the source of pathogenic resistance genes which is a natural phenotype found in almost all bacterial species (Graham, 2015; Jiang *et al*., 2017; Cox & Wright, 2013). Microbial communities have been on earth for billion years and antibiotic resistance genes have been around even before the introduction of antibiotics. These natural resistance genes protect their producers from the bioactive compounds they produce (Fair & Tor, 2014; Genilloud *et al*., 2014). Antibiotic resistance can also arise from chromosomal gene mutations and genetic transfer between bacterial species via horizontal gene-transfer HGT which allows bacteria to acquire

antibiotic resistance through conjugation with the help of plasmids and transposons, through transformation and acquisition of naked DNA from the environment and through transduction by bacteriophages. Additional resistance mechanisms include efflux pumps in the bacterial membrane that eject antibiotics out of the cell, exclusion of antibiotics by the cell membrane, intra-cellular modification and deactivation of antibiotics (Allen *et al*., 2014; Marti *et al*., 2014; Kasanah & Hamann, 2004).

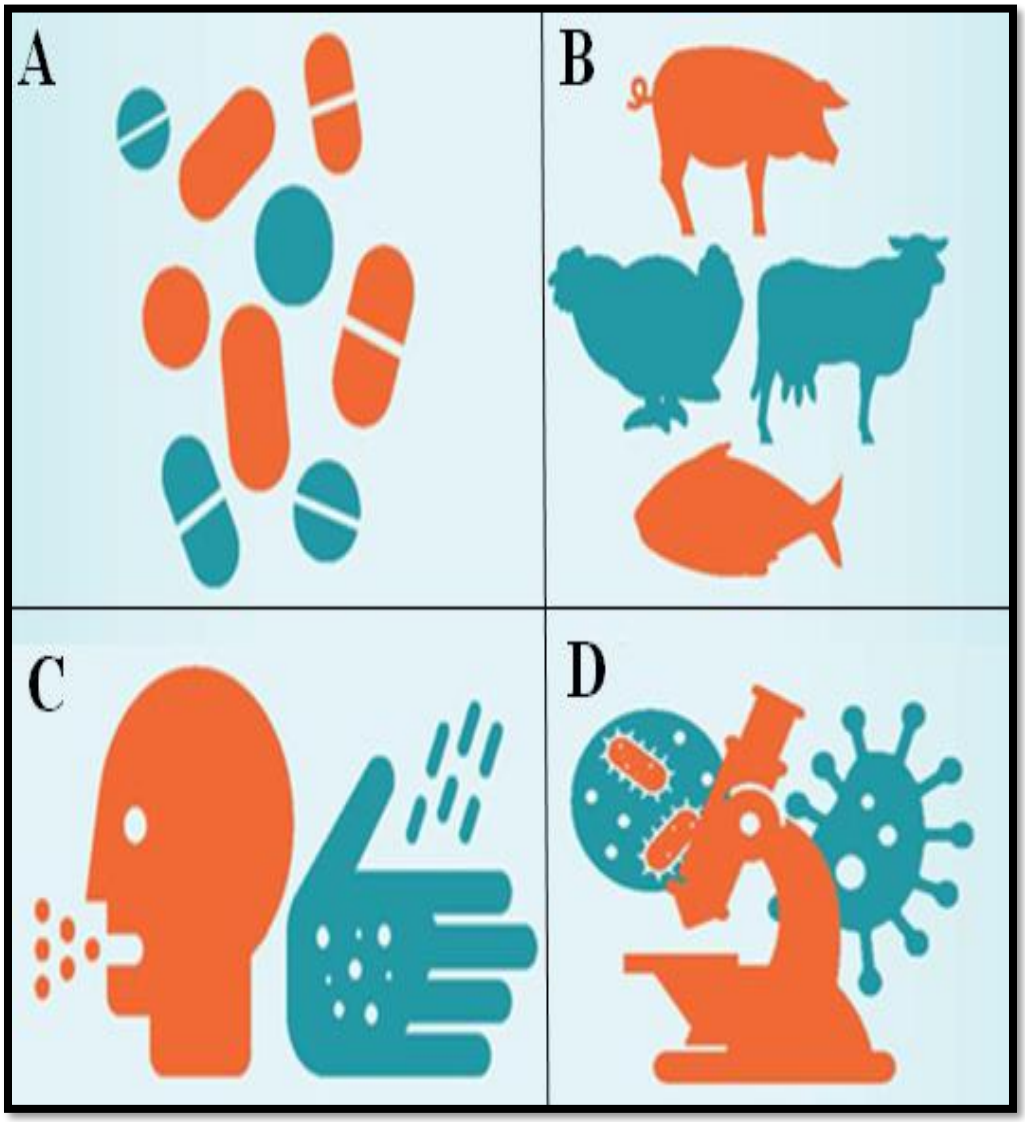

Figure 1.3: Common causes of antibiotic resistance. A; over-prescription of antibiotics, B; use of antibiotics in agriculture and fish farming, C; inappropriate sanitation practices and personal hygiene, D; lack of new antibiotics discoveries. Adapted from https://www.cdc.gov/globalhealth/infographics/antibiotic_resistance_global_threat.htm

1.5 Prevention and control strategies

For many years, antibiotic resistance has been considered a 'problem of individual patients' and all efforts were focused on the prevention of acquisition of resistance during therapy. It was not until recent years that antibiotic resistance was recognized as a 'problem of hospitals' and specific 'antibiotic policies' were proposed to decrease local selection of resistant organisms and to prevent patient-to-patient transmission. Hand hygiene can be used to reduce antimicrobial pressure in the community. Social media can promote hand hygiene activities and behavior change. Hand hygiene improvement strategies include, the application of alcohol-based hand-rub in hospitals, work places and schools as well as implementing compliance monitoring and feedback (Pires *et al*., 2017; Baquero *et al*., 2014).

Despite the fact that antibiotic resistance is a public health concern, the surveillance for this phenomenon in environmental settings is limited which might be due to the fact that antibiotic concentrations in non-clinical settings are very low therefore it is the deployment of antibiotics in a clinical setting that is crucial and emphasizing the importance of applying combined-synergistic therapy in enhancing the control of resistance and treatment efficiency (Bollenbach, 2015; Gammon, 2014). Policy makers; governments, regulatory bodies, hospital administrators and public health experts have a responsibility to raise awareness and integrate solutions to antimicrobial resistance. International groups and agencies including WHO, Centers for Disease Control and Prevention (CDC) and World Health Assembly emphasized the need for implementing immediate global actions against antimicrobial resistance; as the number of pharmaceutical companies is declining, human and financial burden of antimicrobial resistance is rising (Keown *et al*., 2014; Marti *et al*., 2014). These agencies proposed numerous solutions with many countries adopting new strategies to ensure the appropriate use of antibiotics through responsible prescription so that antibiotics are only used when indicated. An appropriate use of existing antibiotics is a must to ensure long term effective treatment for bacterial infections. It should be noted that the use of antibiotics as growth-promoters in animal farming has been banned in Europe since 2006 (Bell *et al*., 2014; Meek *et al*., 2015). Potential prevention approaches include, prolonging the therapeutic life of current antimicrobials via drug cycling; replacing an antimicrobial belonging to one class with one or more belonging to different classes. Studying the possibility of reducing the presence of antibiotic resistant genes in wastewater and the development

of vaccines against nosocomial infections (Holmes *et al*., 2016; Bennett, 2008). To prevent a global human threat, it is imperative to discover new sources for the production of novel antibacterial compounds (Cox & Wright, 2013). There are various challenges facing microbial natural drug discovery. Examples include: (1) unculturable microbial derived environmental samples, (2) failure to identify or activate small molecular bioactive metabolites from silent bacterial genetic pathways, (3) reduced bioactivity of natural compounds post-initial screening or low stability which hinder their applications as clinical drugs and (4) probability of toxicity to the patient (Challinor & Bode, 2015; Trautman & Crawford, 2016).

1.6 The Marine Environment

For centuries, humans have realized the many bioactive properties of the sea and its inhabitants. The marine environment is a complex ecosystem with oceans covering 70% of the world's surface and includes open-ocean ecosystems, thermal vent ecosystems, kelp forests, mangroves and coral reefs (Titilade & Olalekan, 2015; Kasanah & Hamann, 2004). The marine environment holds fascinating biodiversity of life forms with more than 400,000 species including, fish, tunicates, ascidians, corals, bryozoans, mollusks, sea slugs, sponges, algae and bacteria (Doshi *et al*., 2011; Abad *et al*., 2011; Darabpour *et al*., 2010).

The marine environment, also called the “Medicine chest of the new millennium”, is an untapped source of novel compounds of great diversity. Various screening programs were developed worldwide with the aim of isolating new compounds and biomolecules with more than 15,000 natural compounds having been discovered to date (Penesyan, 2010; Debbab *et al*., 2010).

The aquatic environment is the home of numerous bioactive molecules and natural metabolites such as; proteins, peptides, polysaccharides, sterols, antioxidants, polyunsaturated fatty acids, polyphenols, probiotics, terpenoids, enzymes, vitamins, minerals and pigments which makes this fascinating environment a precious source of both economic and scientific importance (Simmons *et al*., 2005; Biswas *et al*., 2016). Squalamine is an amino-sterol drug from the dogfish shark with strong antimicrobial and anti-cancer activities (Skropeta & Wei, 2014). Fish and related products are a major source for the food industry and provide 16% of the world’s protein requirements and are rich of proteins, fatty acids, minerals and vitamins (Donia & Hamann, 2003).

Enzymes derived from marine organisms have various applications and have been used for years in biotechnology due to their high activity and salt tolerance. Marine enzymes include lipase, chitinolytic enzymes and transglutamase. Marine compounds are a promising source of novel drugs with various applications proven to be active against cancer cell lines, HIV and encompass anti-bacterial, anti-viral, anti-fungal, anti-protozoal, anti-tuberculosis and analgesic activities (Dufourcq *et al*., 2013; Colwell, 2002; Mayer *et al*., 2010).

It is widely believed that the unique properties of marine organisms in comparison with terrestrial ones is largely due to the complex marine environmental niches they inhabit (Ruiz *et al*., 2010). These marine niches are characterized by extreme conditions including physio-chemical and biological properties that favor the production of diversely active secondary metabolites and the production of novel compounds as a means of adaptive survival mechanism to the hostile marine habitat (Suleria *et al*., 2015; Abad *et al*., 2011). Marine microbes survive extreme conditions in the absence of light, low levels of oxygen and extremely high pressure. Also, anaerobic conditions and below 0◦C temperatures in the deep-sea and highly acidic conditions with temperatures over 100◦C in hydrothermal vents not to mention competing for space and marine fouling (Donia & Hamann, 2003). All these factors have contributed to the production of diverse array of bioactive metabolites (Katz & Baltz, 2016; Doshi *et al.,* 2011). Some of these natural products have no actual metabolic functions within the producing organisms and are more of a protective agent against predators like the non-protein (palytoxins) produced by dinoflagellates which cause shellfish poisoning (Colwell, 2002).

1.6.1 Marine invertebrates

Marine invertebrates encompass a diverse group of inhabitants including, sponges, tunicates, bryozoans and molluscs. Extracts isolated from marine invertebrates can have a wide range of anti-pathogenic properties mainly, antibacterial, antiviral, anti-helminth and anti-fungal (Abad *et al*., 2011; Skropeta & Wei, 2014).

Shrimp and krill encompass omega-3 fatty acids. The health benefits claimed for products of these organisms are various and include treatments for osteoporosis, Paget's disease, reducing the risk of cardiovascular problems and neurodevelopment. Other benefits include the management of

hypertension, arthritis, total blood lipids and improving Vitamin A levels (Donia & Hamann, 2003).

Marine sponges such as; *Theonella swinhoei* are of great importance due to their ability to biosynthesize secondary metabolites like; polyunsaturated fatty acids, peptides, alkaloids, minerals and carotenoids which are active against *Leishmania*, *Neisseria gonorrheae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Candida albicans*, herpes simplex and HIV (Wietz *et al*., 2013; Jamal *et al*., 2006). Also, they are effective anti-cancer agents, and some hold anti-diabetic properties (Laport *et al*., 2009). Studies speculate that bacterial strains closely associated with sponges are the true source of these properties (Manivasagan *et al*., 2013; Gammon, 2014).

Marine snails possess antimicrobial peptides and peptide cono-toxins which are active against fish and sea predators and Gram-positive and Gram-negative bacteria. Ascidians produce a diverse array of metabolites with anticancer properties such as the compound Didemnin B isolated from the ascidian *Tridemnum solidum* (Dolashka *et al*., 2011; Grabley & Thiericke,1999). Bioactive peptides isolated from fermented blue mussels and oyster sauces have been found to significantly decrease hypertension and amino acids of marine molluscs can improve skin wound healing. Bryostatins are polyketides produced by marine bryozoans and demonstrate antagonistic activity against predatory fish. A clinically important analogue of these polyketides is the anticancer drug Bryostatin 1 which exhibits potent cytotoxic activities (Donia & Hamann, 2003; Ridley *et al*., 2008).

1.6.2 Marine algae

For centuries, marine algae and seaweeds have been used as a food source, in traditional medicine and cosmetics. Marine algae possess strong antioxidant properties and an excellent source of nutrients for human health (Cavallo *et al*., 2013). They are rich in β-carotene, polysaccharides, iron, manganese, zinc, phenolic compounds, minerals, antioxidants, proteins and polyunsaturated fatty acids (El Gamal, 2010). Marine algal metabolites are diverse and include macrolides, proteins, polyketides, terpenes and fatty acids. These compounds can have potent antiviral, antibacterial and anti-parasitic activity (Gammone *et al*., 2014).

Red algal species *Sphaerococcus coronopifolius* are strong producers of antibacterial compounds like Diterpene. Anti-plasmodial activity has been detected in methanolic extracts of marine

seaweeds extracted from the west coast of India (Donia & Hamann, 2003). There are several studies about extracts from green, brown and red marine seaweeds as promising antibacterial agents against *Vibrio parahaemolyticus* which causes outbreak infections of vibriosis in fish and shellfish, as well as affecting human health causing gastroenteritis and food poisoning (Habbu *et al*., 2016; Cunha & Grenha, 2016).

The novel compound Isopropoxymethyl benzylis derived, from marine red alga has promising potential in managing blood sugars (Simmons *et al*., 2005). *Ascophyllum nodosum* a brown seaweed species from the northeast coastal region of United States, contains phenolic compounds with antioxidant effects. The brown alga *Pachydicton coriaceum* produces the anti-microbial compound Pachydictol A which is active against *S. aureus*. Also, many marine seaweeds and algae produce alkaloid and polyketide neurotoxins including saxitoxin and brevetoxins (Gammone *et al*., 2014). Marine polysaccharides of red and brown seaweeds such as fucan, agar, carrageenan and alginate have many biological functions as antiviral, anticoagulant, anti-proliferative and antithrombotic agents. Pigments from red and blue-green algae are used as nutraceutical agents and in food colouring (Cavallo *et al*., 2013; Cunha & Grenha, 2016).

1.6.3 Marine microorganisms

The field of microbial marine products is considered a young science. It only started in the 1960s and it was not until the 1980s where its applications were recognized in clinical medicine, drug discovery and biotechnology. Marine microorganisms are genetically diverse and are famous for their production of biologically active secondary metabolites (Figure 1.5) to protect them against predators and in stressful environmental conditions such as biofilm formation. Marine microbial metabolites include polyphenols, peptides and phlorotannins (Prasad *et al*., 2014; Choi *et al*., 2015).

Several marine bacteria are known for their slow growth in their natural habitat which makes optimum incubation time an important factor for their cultivation in laboratory settings (Wu et al., 2015; Saleem & Iqbal, 2015). Researchers have discovered a wide range of marine metabolites which have been successfully developed by both the biotechnology sector and the pharmaceutical industry. Marine bacterial products applications include, bioremediation, food industry, bio-fuel production, anti-fouling, cosmetics, anti-cancer drugs and antibiotics (Wietz *et al*., 2013; Xiong *et*

al., 2013; Gerwick & Fenner, 2013). Marine *Pseudomonas* species are famous for their production of clinically important secondary metabolites. The first antibiotic derived from marine bacteria was 2-(3', 5'- dibromo-2'-hydroxyphenyl)-3,4,5-tribromopyrrole from *Pseudomonas bromoutilis*. The antibiotic showed potent activity towards Gram-positive bacteria. A heat stable antimicrobial compound from the culture supernatant of *P. aeruginosa* PG-01 isolated from the Persian Gulf showed potent activity against pathogenic Gram-positive bacteria including MRSA (Manivasagan *et al*., 2013; Darabpour *et al*., 2010). Microbial marine secondary metabolites of importance include, macrolide antibiotics, glycopeptides, anticancer agents, enzymes and pigments (Suleria *et al*., 2015; Egan *et al*., 2008). The full genome sequence of the marine actinomycete *Salinispora tropica* revealed 19 secondary metabolic biosynthesis gene clusters encoding different natural metabolites such as; terpenoids and the anticancer agent (Salinosporamide A). The bacterium also produces phylogenetically distinct polyketide synthases and encompasses diverse polyketide biosynthetic pathways, including type I polyketide synthases, type II and type III polyketide synthases (Gulder & Moore, 2009; Udwary *et al*., 2007).

Marine *Bacillus* sp. such as *B. marinus* and *B. amyloliquefaciens* SH-B10 produce potent antibacterial and antifungal activity against *S. aureus* and plant associated fungal pathogens (Abad *et al*., 2011; Biswas *et al*., 2016). Compounds produced by marine *Myxobacteria* are active against cancer cell lines and Gram-negative pathogens (Peláez, 2006). Some species of marine *Pseudoalteromonas* produce low and high molecular weight compounds that are active against *P. aeruginosa*, methicillin-resistant *S. aureus, B. subtilis, E. coli* and *Enterococcus* species. The marine bacterial strain *Marinomonas mediterranea* produces antibacterial activity against resistant nosocomial bacterial strains including, *Pseudomonas* sp. and *S. aureus* (Dufourcq *et al*., 2013; Egan *et al*., 2008). Organic extracts of marine bacterial cultures isolated from marine sediments have a broad spectrum of anti-bacterial, as well as antifungal activity and identification of these inhibitory compounds has led to the discovery of novel marine bacterial alkaloids (Wu *et al*., 2015). Many marine bacterial species produce novel carotenoid pigments with antioxidant activities and medicinal properties against cardiovascular diseases (Blunt *et al*., 2013).

Heterotrophic cold-loving marine bacteria are important due to their production of enzymes like lipase, urease, gelatinase and DNase (Manivasagan *et al*., 2013). Marine bacteria from deep sea hydrothermal vents produce heat stable and acid resistant α-amylase enzymes with promising biotechnological potential (Cragg & Newman, 2013). Other examples of marine derived bacterial

enzymes include agarase, carrageenase and proteases isolated from *Colwellia* and *Marinomonas* species as well as DNA polymerases and DNA ligases from *Thermococcus* and *Pyrococcus* (Imhoff *et al*., 2011). Bacteria isolated from the South China Sea produce novel alkaline protease enzymes with thermal and chemical stability and high activity towards a variety of salt concentrations and organic solvents with potential applications in the detergent and food industry as well as in the treatment of wastewater (Shindo & Misawa, 2014; Gammone *et al*., 2014).

Marine fungal species are still largely underexplored, approximately 700 novel compounds were discovered to date with some exhibiting bioactive properties in particular against Gram-positive bacteria (Navarri *et al*., 2016; Debbab *et al*., 2010). Examples of marine fungal species include, *Penicillium*, *Aspergillus* and *Fusarium* (Habbu *et al*., 2016) and are famous for their production of antibiotics, polysaccharides, polyketides, alkaloids and antioxidants (Figure 1.4) (Rateb & Ebel, 2011; Debbab *et al*., 2010). Marine Fungal secondary metabolites have anti-proliferative activity and can inhibit methicillin-resistant *S. aureus* (Müllera & Wink, 2014; Xiong *et al*., 2013).

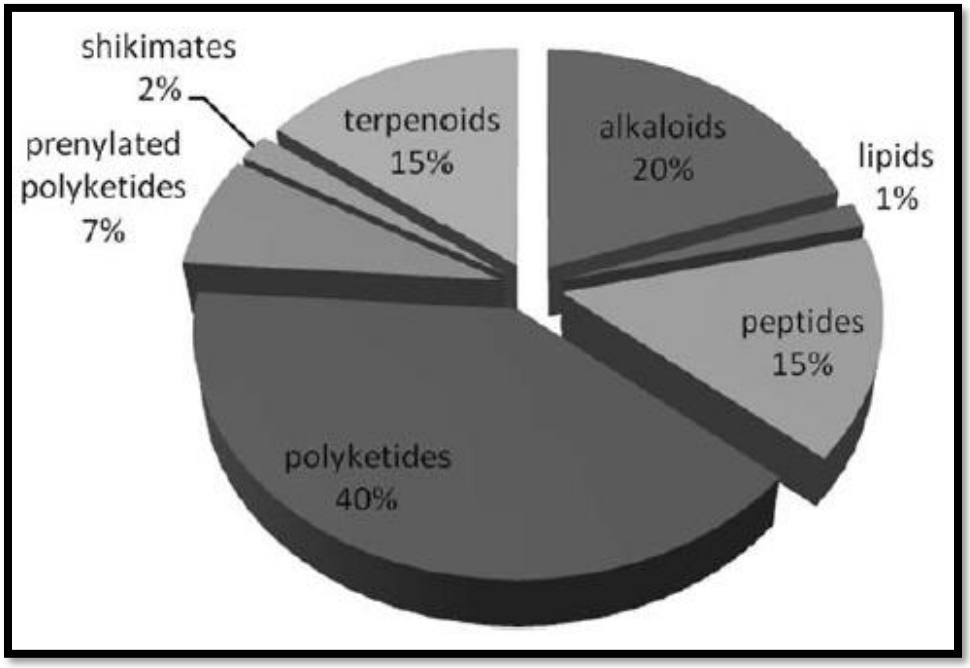

Figure 1.4: Examples of marine-derived fungal compounds (Rateb & Ebel, 2011)

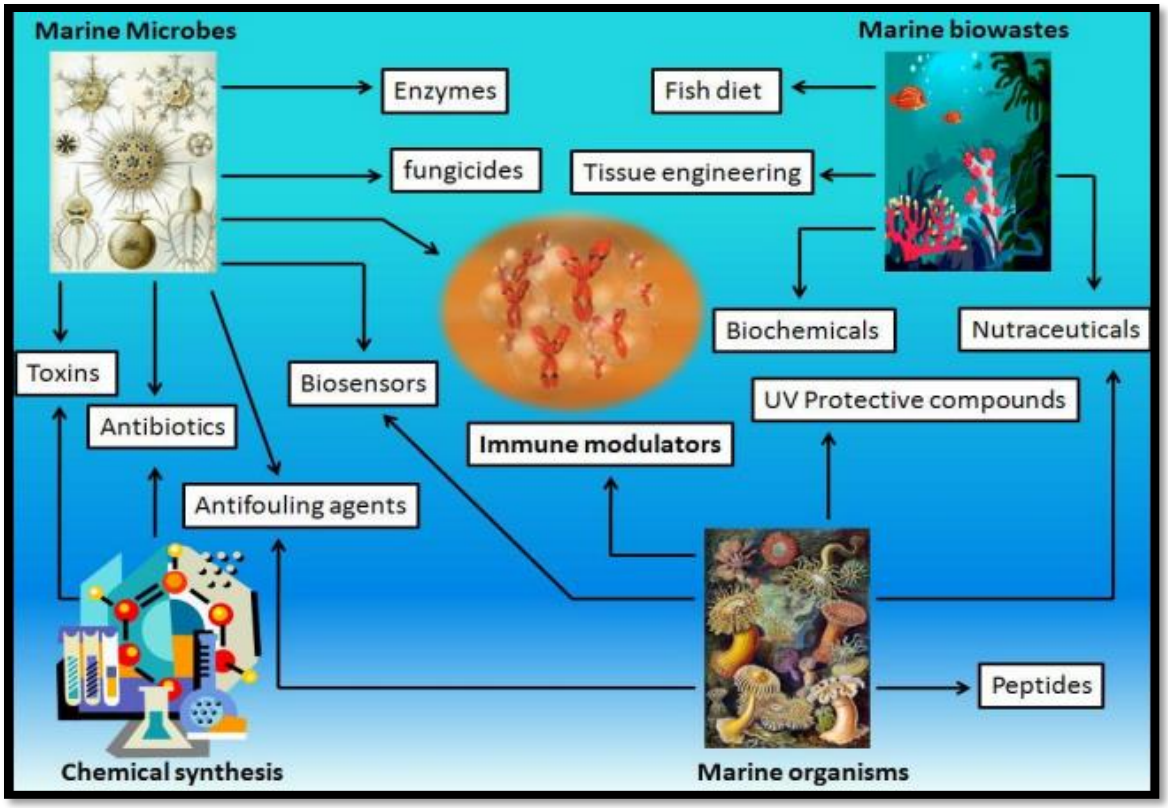

Figure 1.5: Diversity of marine microbial metabolites and their various applications (Bhatnagar & Kim, 2010).

1.7 The genus *Serratia*

1.7.1 Classification

Serratia species of the family *Enterobacteriaceae* are rod shaped opportunistic Gram-negative bacteria of the c subclass of *Proteobacteria* and are motile, psychrophilic and facultatively anaerobic (Grimont & Grimont, 1978; Akele *et al*., 2015). They are named after the Italian physicist Serafino Serrati. They are ubiquitous; inhibit a variety of different environmental niches such as; water, soil, plants as well as insects and animals with some associated with food spoilage. Some examples of *Serratia* include, *S. fonticola*, *S. plymuthica*, *S. marcescens* and *S. grimesii* (Kamble & Hiwarale, 2012; Williamson *et al*., 2005; Weise *et al*., 2014).

In laboratory settings, *Serratia* species can grow on solid media at temperatures ranging from 20°C to 37°C while, in liquid media from 5 °C to 40 °C with optimum pH values of 5- 9. *Serratia* grow in many complex growth media, these include LB, PDA and NA (Stock *et al*., 2003; Houdt *et al*., 2007; Houdt *et al*., 2014; Stancu, 2016). The production of antimicrobial compounds by *Serratia* is carbon source dependent and highly induced in the presence of nutrients like organic acids and sugars and temperature-regulated with enhanced production at lower temperatures since seasonal variations are a major factor in influencing bacterial metabolic activity (Matilla *et al*., 2016a; Masschelein *et al*., 2015b; Adam *et al*., 2016).

Some strains of *Serratia*, in particular *S. marcescens* are human pathogens and the causative agents of contamination in hospital medical devices. *S. marcescens* associated with nosocomial infections cause pneumonia, septicemia, meningitis, endocarditis and urinary tract infections (Vleesschauwer & Hofte, 2007; Ashelford *et al*., 2002; Bonnin *et al*., 2017; Uğras *et al*., 2014; Ersoz *et al*.,2014).

1.7.2 The red pigment prodigiosin

For many years now, natural pigments from microbial sources have been studied for their various biological activities. These include anti-oxidants, antifungal and immuno-suppressive properties. Prodigiosin is a red non-diffusible, water-insoluble pigment bound to the bacterial cell envelope of some strains of *Serratia* such as *S. plymuthica*, *S. marcescens* and *S. rubideae*. However, the pigment is soluble in organic solvents such as methanol (Elkenawy *et al*., 2017; Jafarzade *et al*., 2013; Darshan & Manonmani, 2015; Grimont & Grimont, 1978).

Prodigiosin is an alkaloid secondary metabolite with colours ranging from dark red to pale pink notably on nutrient agar. The majority of reported *S. marcescens* isolates are of clinical origins and appear non-pigmented in comparison to environmental strains. It is strongly believed that it is temperature related since the optimal temperature for the production of prodigiosin is 28°C (Weise *et al*., 2014; Su *et al*., 2016; Faraag *et al*., 2017). The biosynthesis of prodigiosin is controlled by numerous environmental and physiochemical factors including temperature, oxygen and pH with maximum production yields achieved in the absence of light. The availability of nutrients in media composition like carbon, nitrogen, inorganic phosphate and salts can influence the production of prodigiosin and a number of selective broth media are used for the production of the pigment. These include marine broth, nutrient broth, peptone glycerol broth and sesame seed broth (Darshan & Manonmani, 2015; Vleesschauwer & Hofte; 2007).

The structure of prodigiosin includes three pyrrole rings with two linked together and the third ring attached to a methene forming a pyrrolopyrrole-ethene linkage (Moons *et al*., 2006; Ibrahim *et al*., 2014). The production of prodigiosin is controlled by a cluster of operonic genes called *pigA-O* (Ruiz *et al*., 2010; Elkenawy *et al*., 2017). Prodigiosin appears in the later stages of bacterial growth with no obvious physiological function. Nevertheless, studies speculated on the true biological functions of prodigiosin. These include, acting as an overflow for metabolic cellular waste products in the producing strains, contributing to surface adherence and enhancing bacterial dispersal while other studies claim that it might act as a sink for excess proline such as in *Streptomyces* (Harris *et al*., 2004; Fineran *et al*., 2005b).

The pigment displays anti-malarial, more general anti-protozoal activities, anti-fungal activities, and a promising potential as an anti-cancer agent due to its potent apoptotic activity in T and B lymphocytes but low cytotoxicity towards normal cells (Faraag *et al*., 2017; Bentley, 1999; Slater

et al., 2003). Prodigiosin shows bacteriostatic effects with anti-bacterial activity against numerous pathogenic strains. These include, *E. coli*, *E. faecalis*, *S. pyogenes* and *Acinetobacter* species (Lapenda *et al*.,2015; Wang *et al*., 2016). Prodigiosin extracts purified from *S. marcescens* IBRL USM 84, *Serratia marcescens* B2 and *S. marcescens* B10 VKM are active against *S. aureus, P. aeruginosa, B. subtilis, B. cereus, salmonella, Shigella, C. albicans, C. utilis, Cryptococcus* as well as algal blooms (Kimyon *et al*., 2016; Thomson *et al*., 2000; Pore *et al*., 2016).

1.7.3 The commercial and biotechnological applications of *Serratia*

Serratia produce commercially important compounds and enzymes such as lipases, serralysin, chitinases, nucleases, protease, haemolysin and amylases. Some strains of *Serratia marcescens* secrete chitinase B which is characterized by high thermal stability. It is strongly believed that enzymatic production in *Serratia* is due to their ability to inhabit various environmental habitats (Akele *et al*., 2015; Abdou, 2003; Harris *et al*., 2004; Williamson *et al*., 2005).

There is a great interest in the role of *Serratia* as cost-effective and environmental-friendly bioremediation agents. *S. marcescens* B742 synthesizes protease and chitosanase and hydrolyzes the proteinsin SSP protein produced by shrimp shell wastes into water-soluble protein hydrolysates (Gaeseidnes *et al*., 2003; Zhang *et al*., 2014).

Serratia isolated from soil and water samples encompass unique enzymatic activity and can degrade carboxylic acids (nitriles). *Serratia* sp. ISTVKR1 biodegradable activity include various chemical compounds and contaminants including organophosphorus pesticides, methyl parathion and p-nitrophenol (Gupta and Thakur, 2015; Alag *et al*., 2015). *Serratia* strains isolated from petroleum-contaminated sites in Norway coastline produce hydrocarbon-degrading activity with great biotechnological potential in the remediation of oil and petroleum spills. A novel non-pigmented strain of *Serratia* isolated from a river in India can hydrolyse urea to ammonia (Ashelford *et al*., 2002; Frankowski *et al*., 2001).

There are numerous studies regarding the important role of *Serratia* as bio-control agents in agricultural crops management including strawberry, cauliflower and olives. *S. plymuthica* A30 shows potent activity against the bacterium pathogen *Dickeya solani* that cause blackleg and soft rot in potato (Petersen & Tisa, 2013; Matilla & Salmond, 2014; Czajkowski & Wolf, 2012). *Serratia* strains used as environmental bio-control agents include, *S. proteamaculans* and *Serratia*

sp. ANU101 which produce various compounds including the antifungals haterumalides which were the first polyketides to be discovered in *Serratia* (Adam *et al*., 2016; Stock *et al*., 2003; Lim *et al.*, 2015). The novel strain *Serratia marcescens* B4A produces potent antifungal compounds and inhibit the growth of insects and plant pathogens such as *Rhizoctonia solani* and *Alternaria raphanin*. The following strains of *Serratia*, *Serratia marcescens*, *Serratia plymuthica*, *Serratia* sp. SY5, Serratia *fonticola* AU-P3 and *Serratia fonticola* DSM 4576^T are plant growth promoting bacteria. They enhance crop yields and ecological balance in the agroecosystem by facilitating the uptake of nutrients from the environment. They also produce secondary metabolites such as siderophores and phytohormone and protect the plants against pathogenic infections (Zarei *et al*., 2011; Matilla *et al*., 2016b; Lim *et al*., 2015).

Some strains of *Serratia* including, *Serratia plymuthica* HRO-C48 produce the halogenated secondary metabolite pyrrolnitrin which is a promising agricultural fungicide (Cleto *et al*., 2014; Liu *et al*., 2007; Bhadra *et al*., 2005). *Serratia nematodiphila* DSM 21420^T is a biological pest control agent and produce potent insecticidal Sep proteins (SepA, SepB, SepC). A full genome sequence of the strain showed gene clusters encoding enzymes contributing to antimicrobial production (Kwak *et al*., 2015). The following strains of *Serratia* including, *S. plymuthica* 4Rx13, *S. marcescens* Db11, *S. odorifera* DSM 4582 and *S. plymuthica PRI-2C* produce volatile organic compounds VOCs including dimethyl trisulfide, sodorifen and methanethioland terpenoids (Popova *et al*., 2014; Su *et al*., 2016; Neupane *et al*., 2012). These compounds have cytotoxic broad bacteriostatic inhibitory activity against various pathogenic bacteria and fungi, fruit flies and nematodes (Dang *et al*., 2015; Schmidt *et al*., 2017).

1.7.4 *Serratia* a novel source of antimicrobial compounds

Serratia produce novel secondary metabolites with potent antibacterial (Table 1.1), anti-fungal as well as anticancer activities (Matilla *et al*., 2016a). Some strains of *Serratia* have a highly species-specific secretion-system (type VI) also known as T6SS which enables the production of broad-spectrum bioactive compounds. This system facilitates the production of antibacterial toxins and self-protecting bacteriophage contained proteins that contribute to virulence against competitors and even related *Serratia* strains (Petersen & Tisa, 2013; Hellberg *et al*., 2015; Fritsch *et al*., 2013).

The production of bioactive secondary metabolites in *Serratia* is due to quorum sensing (QS) (Chernin *et al*., 2011; Su *et al*., 2016). QS regulates gene expression in many Gram-negative bacteria in response to environmental selective pressure like the depletion of nutrients and influences population density by the production of *N*-Acyl homoserine lactone (AHL) molecules (Liu *et al*., 2011; Thomson *et al*., 2000; Wencewicz, 2016). AHL are intercellular auto-inducer diffusible signaling molecules biosynthesized by the enzyme LuxI and regulates the production of antimicrobials, antibiotics, enzymes and plant growth promoting compounds as well as contributing to motility, sporulation, virulence and biofilm formation (Weise *et al*., 2014; Sanchez *et al*., 2010b). There are various studies regarding the antimicrobial metabolites of *Serratia* (Table1.1). The culture supernatant of *Serratia marcescens* 2170 have strong cytotoxic activity against cancer cell lines (Coulthurst *et al*., 2004). Also, *Serratia* sp. strain American Type Culture Collection 39006 produces the broad spectrum β-lactam antibiotic Carbapenem. *S. marcescens* 274 and *Serratia* 39006 secrete haemolysin, prodigiosin (Montaner *et al*., 2000; Fineran *et al*., 2005a).

Some strains of *S. marcescens* such, as *S. marcescens* strain NSK-1and *S. marcescens* IBBPo15 produce the lipopeptide compounds, serrawettins synthesized by polyketide synthases. Serrawettins are broad spectrum antibacterial bio-surfactants and potent anticancer agents against T-cell leukemia and Burkittis lymphoma (Su *et al*., 2016; Penesyan, 2010).

Recent research regarding *S. plymuthica* A153 and *S. marcescens* MSU97 revealed the production of the antifungal compound antioomycete, the anticancer agent haterumalide and the antibiotic andrimid. The latter inhibits the growth of *Salmonella enteritidis*, *Yersinia enterolitica*, *Vibrio harveyi* and *Enterococcus* (Matilla *et al*., 2016b; Uğras *et al*., 2014; Martinez *et al*., 2015).

S. grimesii and *S. proteamaculans* produce anti-cancer metabolites active against human larynx carcinoma (Malheiros *et al*., 2015; Matilla *et al*., 2016a). Some *Serratia* produce the exoenzymes oocydin A and bacteriocins (Williamson *et al*., 2006; Sanchez *et al*., 2010 b). *S. marcescens* Db10 secretes the antibacterial toxins Ssp1 and Ssp2 and produces self-resistance proteins as a protection mechanism from its own toxins (Cleto *et al*., 2014; Matilla & Salmond, 2014). Strains of *Serratia* such as *S. plymuthica*, *Serratia* sp. strain V4 and *S. plymuthica* RVH1 produce zeamine antibiotics which have broad spectrum bactericidal activity against multidrug resistant bacteria and yeast. Zeamines cause membrane permeabilization through hydrophobic interactions with phospholipid

layers and have cyto-toxic activities against human cancer cell lines (Ovadis *et al*., 2004; Masschelein *et al*., 2015a; Masschelein *et al*., 2013).

Table 1.1: Examples of *Serratia* antimicrobial metabolites

Strain	**Metabolite(s) and antimicrobial activity**	**Reference**
S.marcescens IBBPo15	Serrawettins: Broad spectrum antibacterial compounds	(Penesyan, 2010)
S. plymuthica A153	Haterumalide: Inhibit bacterial protein biosynthesis	(Matilla *et al*., 2016b)
Serratia sp. strain American Type Culture Collection 39006	Carbapenem: Inhibit bacterial cell wall synthesis	(Montaner *et al*., 2000)
S. plymuthica RVH1	Zeamines: Affect the integrity of bacterial cell membrane.	(Ovadis *et al*., 2004)
Most *S. marcescens* strains	Prodigiosin: Bacteriostatic anti-bacterial activity, affect the bacterial cell membrane and decrease the cells respiration activity.	(Faraag *et al*., 2017; Bentley, 1999; Wang *et al*., 2016; Thomson *et al*., 2000; Pore *et al*., 2016; Danevcic *et al*., 2016)

1.7.5 Antimicrobial polyketides

The discovery of polyketides was by James Collie in 1883 but it was not until the 1950s that the field of polyketide research was established by Arthur Birch after his work on the aromatic polyketide (6-methylsalicylic acid) from *Penicillium patulum* (Snyder *et al*., 2003; Tang *et al*., 2004; Gallo *et al*., 2013).

Polyketides are structurally diverse natural secondary metabolites. They are synthesized by a family of multi-modular enzymes known as polyketide synthases (PKSs) from the oligomerization of carboxylic acids (Korman *et al*., 2010; Donadio *et al*., 2007; Kwan & Schulz, 2011). PKSs consist of separate enzymatic domains. These include, a ketosynthase (KS), an acyl carrier protein (ACP) and an acyltransferase (AT) (Gerc *et al*., 2012; Bedford *et al*., 1996). These domains are responsible for the incorporation of a specific building block or intermediate product leading to the formation of the final product in an 'assembly-line' fashion (Mcdaniel *et al*., 1999; Wagner *et al*., 2016; Sundaram *et al*., 2015).

The unique biodiversity of polyketides is largely due to the order of the modules which determines chemical pathways and the structure of the final product. The biosynthesized polyketides can go through a secondary modification step in the post-PKS process where their chemical structure is further oxidized or methylated by O-methyl transferase (Zhang *et al*., 2009; Wiesmann *et al*., 1995; Cheng *et al*., 2009; Callahan *et al*., 2009). Horizontal gene transfer also contributes to the biodiversity of polyketides through exchanged or altered modules leading to alternate tailoring enzymes and the production of new polyketides (Hojo *et al*., 2015; Bachmann, 2016; Matilla *et al*., 2015).

For many years, there has been a great interest in bacterial PKS genes and the fascinating metabolites they encode (Dutta *et al*., 2014; Nair *et al*., 2012). PKSs genes are widely distributed within bacterial communities and organized in gene clusters within bacterial genomes but not necessarily as single operons. PKSs derived secondary metabolites represent a vast collection of bioactive products with some studies emphasizing their importance in the survival and pathogenicity of the producer strains (Shen, 2003; Staunton & Weissman, 2001).

PKS genes have been found in many strains of *Serratia* in particular *S. plymuthica*, *S. marcescens* and *S. odorifera.* These genes are responsible for the production of novel compounds with bioactive functional and structural diversity and applications in medicine and clinical practices.

These include anti-bacterial, anticancer, anti-fungal, anti-parasitic and immunosuppressive properties (Awodi *et al*., 2017; Chan *et al*., 2009; Snyder *et al*., 2003). The pharmacological importance of these compounds and their analogues exceed $10 billion per year. These include, tetracyclines, rapamycin, lovastatin, streptomycin and amphotericin (Kwan & Schulz, 2011; Kodama *et al*., 2005).

Bacterial PKSs can be categorized into three different types and share the same functionality but differ in structure. Type I PKSs are multifunctional enzymes organized into modules, located in a single protein and regulate the synthesis of different non-similar polyketide products and account for approximately one-third of clinical drugs in the pharmaceutical market. Examples include, erythromycin and epothilone (Menzella *et al*., 2010; Shen, 2003; Nair *et al*., 2012). Type II PKSs are composed of complex single module proteins but with separate enzymatic activities and synthesize aromatic polycyclic polyketides like tetracenomycin and doxorubicin (Eichholz *et al*., 2012; Sanchez *et al*., 2010b). Type III PKSs consist of a single active site enzyme that controls the biosynthesis of the final product like the aromatic polyketides flavolin and tetracycline (Neupane *et al*., 2012; Tang *et al*., 2004).

1.8 Aims

The rise of antibiotic resistance is a serious health problem affecting many people around the world and the search of effective antimicrobials is urgently needed to combat this resistance.

Bacterial derived bioactive metabolites have been around for billions of years in environmental habitats as natural means of defense and the majority of clinical drugs currently used are derivatives of these metabolites.

The environment, including the marine environment encompass enormous biodiversity with remarkable natural compounds with great capabilities for drug discovery and the synthesis of antimicrobial agents. Marine bacteria produce natural metabolites with unique biological properties and understanding the biochemical nature of these compounds is the key for future development of new drugs and antibiotics.

Serratia species synthesize a diverse array of secondary metabolites, many of which represent a promising renewable and valuable resource for pharmaceutical industry and research.

The aim of this project was to characterize the secreted antibacterial activity of an environmental isolate of *S. plymuthica* and determine whether there was more than one compound responsible for this activity.

- The specific objectives of this study were:

1- Optimization of secreted antibacterial activity by investigating the influence of different incubation periods, media composition and culture vessels on the production and amount of secreted antibacterial compound(s) by the environmental isolate *S. plymuthica*.

2- Characterization of secreted antibacterial activity by investigating its stability towards storage temperatures, Proteinase K, pH and TWEEN.

3- Estimating the molecular weight of the secreted antibacterial compound(s).

4- Investigating the spectrum of secreted antibacterial activity and if its bactericidal or bacteriostatic

5-Genetic analysis of *S. plymuthica* using (Transposon mutagenesis) and cloning the genomic region(s) that code for antimicrobial activity.

6-Isolation and purification of the secreted antimicrobial compound(s).

7- Structural characterization of the secreted antibacterial compound(s) via NMR and COSY spectrometry.

Finally, the agents of the antibacterial activity were evaluated for potential development as commercial antimicrobial compounds.

CHAPTER 2: MATERIALS AND METHODS

2.1 General methods

2.1.1 Materials

All chemicals, culture media, reagents, enzymes and other materials used in this study were purchased from Sigma-Aldrich (UK) and OXOID, MELFORD, Fisher Scientific (UK), FORMEDIUM and Helena Bio Sciences unless otherwise stated in the text. Manufacturers' instructions were applied on storing and handling of the materials. Media, solutions, tips and glassware were autoclaved at 121^{o}C for 15 mins.

2.1.2 Centrifugation

Centrifugations were run according to manufacturers' instructions and at an appropriate speed and temperature using an Eppendorf™ Minispin bench top centrifuge for small samples (500µl-2 ml) while larger ones measuring up to 50 ml were centrifuged using a Beckman coulter allegra X-12R bench top centrifuge.

2.1.3 Growth media and cultivation conditions

For the purpose of this study, microbial strains were cultured on nutrient agar (NA) plates (28g/L) and in nutrient broth BN (25 g/L) for 18h-24h. *E. coli* strains were grown on Luria-Bertani LB agar plates (Composition per 1L: LB broth 25g and 14g Agar) and in LB broth (25g/L) and incubated at 37°C for 24h. Fungal and yeast strains were grown on YPD agar plates; (Composition per 1 L: 10g yeast extract, 20g peptone, 20g glucose anhydrous and 20g agar) and incubated at 25 °C for 2 days. Additional media used in this study include Brain heart infusion broth BHI (37g/L) and de Man, Rogosa and Sharpe MRS broth (52g /L). Antibiotics [kanamycin (50µg/µl) and ampicillin (50µg/µl)] were added as needed to growth media after cooling to (~55^{o}C) after autoclaving. Incubations were performed aerobically on a shaking plate/shaking water at appropriate temperatures and at a speed of (120 rpm).

2.2 Microbial strains and plasmids

A variety of microorganisms including bacteria, yeast and fungi were used in this study as shown in Table 2.1.

2.2.1 Wild type strain

In 2009, the environmental strain *S. plymuthica* was isolated by Narges Khalaf from the surface of the seaweed *Ascophyllum nodosum* from Hawcraig point at Aberdour beach, Scotland (Figure 2.1).

Figure 2.1: Sample collection site at Hawcraig point, Aberdour beach, Scotland

Table 2.1 Microbial strains and plasmids used in this study

Strain or plasmid	**Characteristics**	**Growth conditions**	**Source**
Serratia plymuthica (P)	Wild-type (Environmental isolate)	NA, NB (RT/18h)	D. Jamieson[1]
Staphylococcus aureus (ATCC 25923)	Indicator strain	NA, NB (37^{o}C/24h)	P. Cyphus[2]
Bacillus cereus variant of *mycoides* (F)	Indicator strain (Environmental isolate)	NA, NB (30^{o}C/24h)	D. Jamieson
Bacillus cereus (ATCC 11778)	Indicator strain	NA, NB (30^{o}C/24h)	P. Cyphus
Bacillus subtilis (ATCC 6633)	Indicator strain	NA, NB (30^{o}C/24h)	P. Cyphus
Pseudomonas aeruginosa	Indicator strain	NA, NB (37^{o}C/24h)	S. IIincheta[3]
Salmonella enterica variant of *Enteritidis* (S) (ATCC 13876)	Indicator strain	NA, NB (35^{o}C/24h)	S. IIincheta
Klebsiella pneumoniae (ATCC 13883)	Indicator strain	NA, NB (37^{o}C/24h)	S. IIincheta
Enterococcus faecalis (ATCC 29212)	Indicator strain	NA, NB (37^{o}C/24h)	S. IIincheta
Serratia marcescens	Indicator strain	NA, NB (30^{o}C/24h)	S. IIincheta

Table 2.1 (continued)

Strain or plasmid	Characteristics	Growth conditions	Source
Saccharomyces cerevisiae (ATCC 4098)	Indicator strain	YPD, YPD broth (37°C/24h)	S. IIincheta
Candida tropicalis LAMB C.tr (Neogen laboratories collection)	Indicator strain	YPD, YPD broth (30°C/24h)	S. IIincheta
Candida krusei LAMB Asp.C76 (Neogen laboratories collection)	Indicator strain	YPD, YPD broth (24°C/24h)	S. IIincheta
Saccharomyces diastaticus Y361	Indicator strain	YPD, YPD broth (37°C/24h)	S. IIincheta
Escherichia coli (ATTC 25922)	Indicator strain	LB, LB broth (37°C/24h)	S. IIincheta
E. coli (BW2020767)	Donor strain Tn5-RL27 (Km^R-*ori*R6K)	LB, LB broth +50 µg/ml Km (37°C/24h)	(Larsen *et al*., 2002)
E. coli S17-1 λpir	Recipient of recombinant plasmids	LB, LB broth (37°C/24h)	(Simon *et al*.,1983)
E. coli EC100D™ pir-116	Recipient of recombinant plasmids	LB, LB broth (37°C/24h)	TransforMax™

Table 2.1 (continued)

Strain or plasmid	Characteristics	Growth conditions	Source
E. coli EC100D™ pir+	Recipient of recombinant plasmids	LB, LB broth (37^{o}C/24h)	TransforMax™
P1-P8	A self-ligated 2000 bp *Eco*RI fragment of *S. plymuthica* chromosomal DNA with Tn5-RL27 (Km^{R}-*ori*R6 K)	LB, LB broth +50 μg/ml Km (37^{o}C/24h)	This study
WP1-WP5	A self-ligated 5500 bp *Eco*RI fragment of *S. plymuthica* chromosomal DNA with Tn5-RL27 (Km^{R}-*ori*R6 K)	LB, LB broth +50 μg/ml Km (37^{o}C/24h)	This study

1-D. Jamieson, School of Energy, Geoscience, Infrastructure and Society, Heriot Watt University, Edinburgh, UK

2-P. Cyphus, School of Energy, Geoscience, Infrastructure and Society, Heriot Watt University, Edinburgh, UK

3-S. IIincheta, School of Energy, Geoscience, Infrastructure and Society, Heriot Watt University, Edinburgh, UK

2.3 Preservation and maintenance of bacterial strains and plasmids

The preservation of microbial strains/plasmids was performed as follows: stock cultures: single colonies were picked from the designated culture plate using a sterile loop and transferred to falcon tubes (50 ml) containing 5 ml of the appropriate liquid medium before incubation for 24h with shaking (120 rpm). The bacterial culture (500 µl) was mixed with an equal volume of 50% (v/v) glycerol. The samples were labeled with a designated name and date and stored at -80^{o}C until needed. Working cultures: kept on appropriate agar plates/ broth at 4 °C.

2.4 Preparation of cell-free culture supernatants (CFCS)

In this study, the preparation of the CFCS was done as follows: the environmental strain (*S. plymuthica*) was grown in 500 ml flasks containing 100 ml of NB broth at 25^{o}C with shaking (120 rpm) for 48h-72h.The bacterial cultures were poured into 50 ml centrifuge tubes and centrifuged at 3000x g for 15mins. The supernatant was collected carefully without disturbing the pellet and placed into new centrifuge tubes. The culture supernatants were sterilized by two methods; heat treatment by placing the samples into a hot water bath (Grant SUB 14) at 70^{o}C for 10mins or by flirtation using a (0.2 µm) cellulose acetate filter.

2.5 Detection of antibacterial activity

The presence of antibacterial activity was assessed by performing a spot agar assay against an appropriate indicator strain as follows: 100 µl of an overnight culture of an appropriate indicator strain was spread onto NA plates using sterile glass beads. The glass beads were removed, and the plates were left to dry at RT for (15-20mins). The desired samples to be tested for antibacterial production were spotted onto the center of the plates (20 µl) and incubated appropriately. A visible clear zone represents an area of growth inhibition and its size was recorded as illustrated in the following figure:

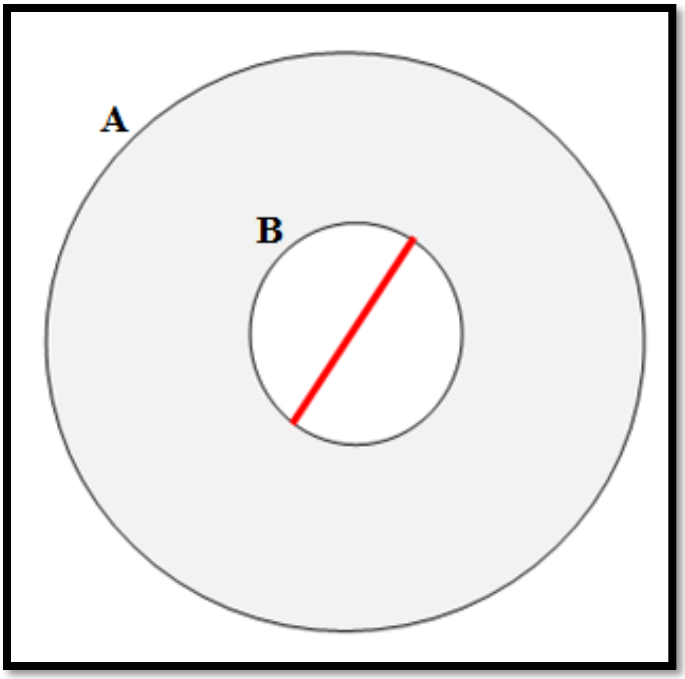

Figure 2.2: An illustration of a spot agar assay. A; bacterial lawn of an indicator strain. B; zone of inhibition is recorded by measuring the diameter of the clear area.

2.6 The influence of incubation period on the production of antibacterial activity

The influence of incubation period on the production of antibacterial activity was tested as follows; Single colonies of a fresh plate of *S. plymuthica* were inoculated in 50 ml centrifuge tubes with 5 ml NB broth and incubated for the following time periods: 24, 48, 72, 96 and 120h. The samples were incubated at 25^{o}C with shaking (120 rpm). Centrifugation was done at 3000x g for 15mins and the supernatant was collected and placed into new centrifuge tubes. The samples were placed into a water bath at (70^{o}C/10mins), then left to cool at RT for 15mins before being ready for analysis.

2.7 The influence of growth conditions on antibacterial production

2.7.1 96 well plates

The influence of growth conditions (culture vessels) on antibacterial production was tested as follows: Single colonies from a fresh plate of *S. plymuthica* were picked out using a sterile loop and individually inoculated into each well of a 96 well- plate (Figure 2.3). Each well contained 300 µl of M.R.S broth media with few wells left cell free as negative controls. The plate was sealed with an adhesive plate semi-permeable membrane and incubated for 72h on a rotary shaker (120 rpm) at 25^{o}C. The plate was placed in a hot water bath (70^{o}C/30mins) in order to eliminate *S. plymuthica* cells. The plate was left to cool at RT for 15mins before being ready for analysis.

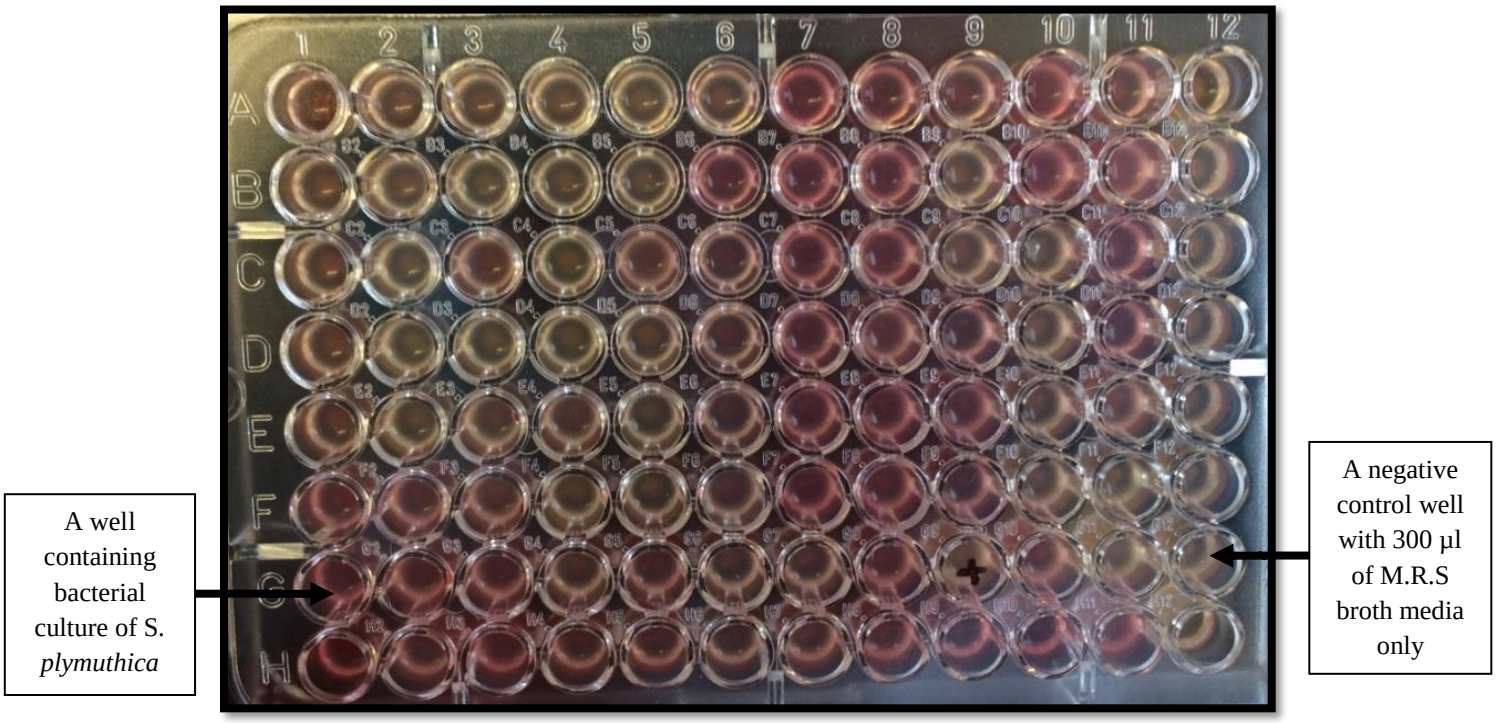

Figure 2.3: Demonstration of a 96 well- plate used to investigate the influence of growth conditions on the production of secreted antibacterial activity. The antibacterial producing strain (*S. plymuthica*) was cultivated in wells containing 300 µl of M.R.S broth media and incubated at 25^{o}C on a rotary shaker (120 rpm) for 72h.

2.7.2 Centrifuge tubes (50 ml)

The antimicrobial producing strain *S. plymuthica* (3 colonies) was cultivated in (Greiner centrifuge tubes, 50 ml; conical (V) bottom) containing 5 ml M.R.S broth media and incubated at 25^{o}C with shaking (120 rpm) for 72h. The samples were spun using a bench top centrifuge at 3000x g for 15mins.The culture supernatant was collected and placed into new falcon tubes. The samples were placed in a hot water bath (70^{o}C/10mins) and left to cool at RT for 15mins before being ready for analysis.

2.7.3 Flasks (250 ml)

The antibacterial producing strain (*S. plymuthica* (5 colonies) was cultivated in 250 ml flask containing 20 ml of M.R.S broth media (Figure 2.4) and incubated at 25^{o}C with shaking (120 rpm) for 72h. Bacterial culture samples were placed into centrifuge tubes. Centrifugation was done at 3000xg for 15mins. The culture supernatant was placed into new centrifuge tubes and placed in a hot water bath for (70^{o}C/10mins) and left to cool at RT for 15mins before being ready for analysis.

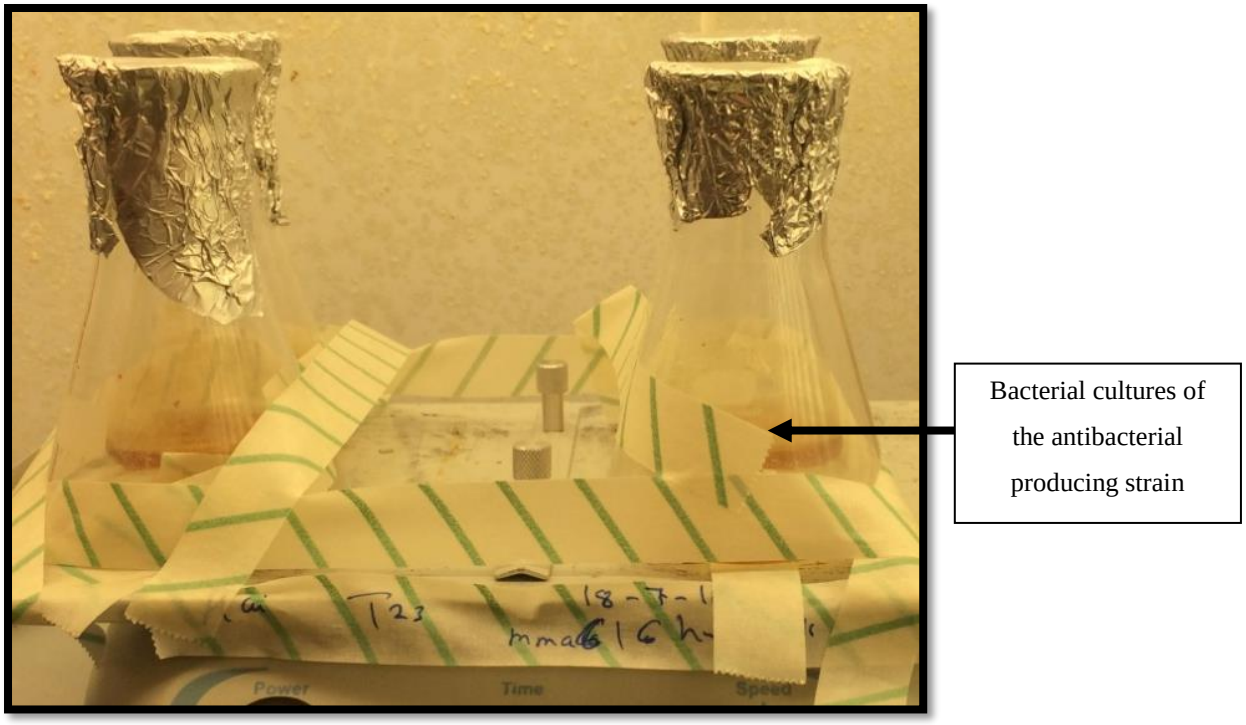

Figure 2.4: Demonstration of 250 ml flasks used to investigate the influence of growth conditions on the production of secreted antibacterial activity. The antibacterial producing strain (S. *plymuthica*) was inoculated in 20 ml M.R.S broth media and incubated at 25^{o}C with shaking (120 rpm) for 72h.

2.8 The influence of cultivation temperatures and media composition on antibacterial production

The influence of cultivation temperatures and media composition on the production of secreted antibacterial activity was investigated as follows: The environmental strain *S. plymuthica* was grown in 250 ml sterilized flasks containing 20 ml of the following broth media: NB broth, LB broth, M.R.S broth and BHI and incubated at (16^{o}C, RT, 30^{o}C and 35^{o}C) with shaking (120 rpm) for 72h. Bacterial culture samples were placed into centrifuge tubes and centrifugation was done at 3000x g for 15mins. The culture supernatant was placed into new centrifuge tubes and placed in a hot water bath (70^{o}C) for 10mins. The samples were left to cool at RT for 15mins before being ready for analysis.

2.9 Effect of heat-treatments on antibacterial activity

The effect of heat-treatments on antibacterial activity was assessed by subjecting the culture supernatant of the antibacterial producing strain (*S. plymuthica*) to the following temperatures (60^{o}C, 70^{o}C, 80^{o}C, 90^{o}C, 100^{o}C) using a water bath for 10mins; heating time did not include the time to attain the desired temperature.

2.10 Stability of antibacterial activity

2.10.1 Effect of storage temperatures on antibacterial activity

The effect of storage on the stability of the antibacterial activity was tested by placing the desired sample at 4^{o}C for 7 days and recording the presence of antibacterial activity against an appropriate indicator strain.

2.10.2 Effect of Proteinase K on antibacterial activity

Enzymatic treatment was performed by incubating Thermo Scientific Proteinase K stock solution (20 mg/ml) with the desired sample before incubation at 37°C for 3h. The desired sample (100 µl) was mixed with (80 µl) of Proteinase K stock solution (20 mg/ml) and sterile distilled water (20µl) and incubated for 3h at 37^{o}C.

2.10.3 Effect of (TWEEN® 20) on antibacterial activity

The detergent; surfactant TWEEN® 20 1% (v/v) was mixed with an equal volume of the desired sample and incubated in a heating block (37^{o}C/3h).

2.10.4 Effect of pH on antibacterial activity

The effect of acidification and alkalization on respective samples was carried out using HCl (1M) (HCL 48ml, distilled water 500ml) and NaOH (1M) (NaOH 20g, distilled water 500ml) to adjust the pH. Acidification was done by adjusting the pH of the desired sample to pH 5 using HCl and alkalization was done by increasing the pH of the desired sample to pH 9 using NaOH. Samples were incubated at 22ºC for 2h with shaking (120 rpm).

2.10.5 Effect of Tris buffer on antibacterial activity

A Tris buffer; [Tris-HCl pH 7.0 (50 mM), NaCl (50mM), Glycerol (5%) and EDTA (2mM)] was added to the desired sample (10:1; Tris-buffer: sample) before incubation at 25ºC for 2h with shaking (120 rpm).

2.11 Properties of antimicrobial activity

2.11.1 Estimation of the molecular weight of the antibacterial compound(s)

Amicon ®Ultra-15 Centrifugal Filter Units (3,000 MWCO) and (10,000 MWCO) were used on respective samples according to manufacturer's instructions.

2.11.2 Measuring the effect of antibacterial activity on bacterial growth

Single colonies from a fresh plate of the indicator strain *B. cereus* were picked out using a sterile loop and inoculated into 50 ml centrifuge tubes with 10 ml of NB and incubated at 37ºC with shaking (120 rpm) for 24h. The overnight culture (100 µl) was inoculated into 10 ml of NB and incubated at 37ºC with shaking (120 rpm) until (OD_{600} ~0.5). The bacterial culture was distributed evenly (500 µl) into centrifuge tubes followed by centrifugation at 3000xg for 10mins. The supernatant was discarded, and the cell pellets were re-suspended in 500 µl of sterile maximum recovery diluent (MRD) PH 7.0; [(Formula/L: peptone (1.0 g), NaCL (8.5g)]. Centrifugation was performed 3 times to thoroughly wash the cells.

2.11.3 Spectrum of antibacterial activity

The inhibitory spectrum of respective samples was investigated against a variety of indicator microorganisms (Table 2.1).

2.12 Transposon mutagenesis

Transposon mutagenesis was performed using plate mating between *S. plymuthica* and *E. coli* (BW2020767) carrying plasmid pRL27.The plasmid contains a mini-Tn*5* element which encodes resistance to kanamycin (Km^R) (Larsen *et al*., 2002; Lorenzo *et al*., 1990).

2.12.1 Conjugation

Plasmid pRL27 was transferred to *S. plymuthica* as follows: single colonies from a fresh plate of the donor strain [*E. coli* (BW2020767)] were picked aseptically and inoculated in 5 ml of LB broth supplemented with 50µg/ml of the antibiotic Kanamycin (Km) while, the recipient strain *S. plymuthica* was inoculated in 5 ml of NB. The bacterial cultures were incubated at 37ºC and 25ºC respectively under shaking condition (120 rpm) for 24h. Then 100 µl of each bacterial culture; donor and recipient were added to 10 ml of LB and NB and incubated as previously mentioned until mid-exponential phase $O.D._{600}$ ~0.8. Then, 1 ml of the donor bacterial culture was concentrated via centrifugation at 3000 x g for 10mins, the supernatant was discarded and the pellet was washed three times with sterile water. The donor strain and the recipient strain were mixed at a 1:1 volume ratio (100µl:100µl) and divided into aliquots of 20 µl and spotted onto NA plates and incubated at RT for 24h.

2.12.2 Transposon selection

After incubation, colonies were scraped off and re-suspended in 10 ml of sterile MRD. Serial dilutions were prepared (1/100, 1/1000, 1/10,000) and cells were plated (including undiluted cells) onto NA plates supplemented with the antibiotics: 50µg/ml Kanamycin (Km) and 50 µg/ml Ampicillin (Amp) so that the former selects for the Tn*5*-transposon while the latter eliminates *E. coli* donor cells and any contaminants. The plates were incubated at RT for 24h/48h in order to isolate *S. plymuthica* transconjugants (Figure 2.5). Km^R colonies were picked out aseptically and sub-cultured onto NA plates+ (50µg/ml Km).

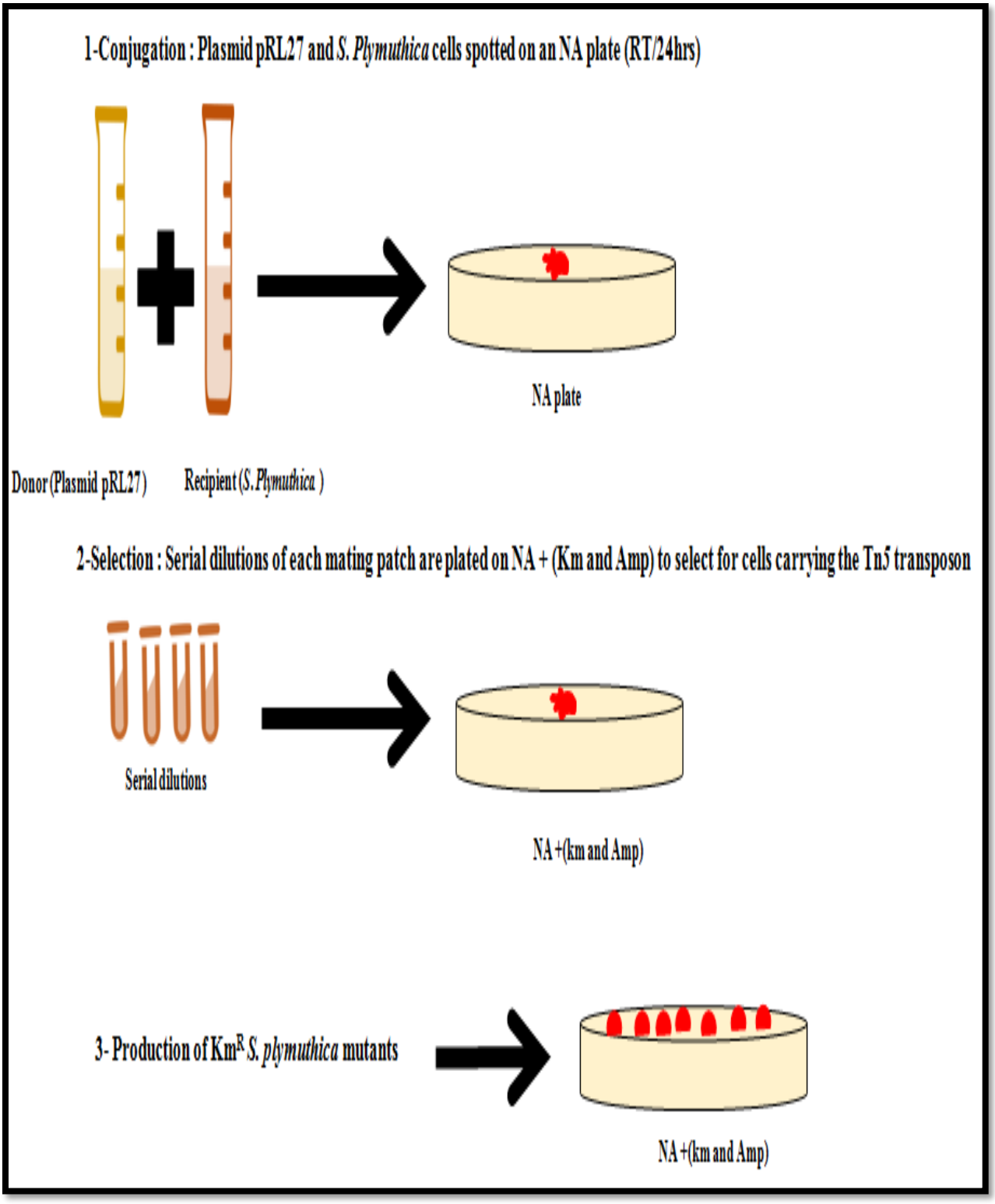

Figure 2.5: Transposon mutagenesis was used in this study between the wild type (*S. plymuthica*) and *E. coli* (BW2020767). This method includes three stages: conjugation, transposon selection and production of *S. plymuthica* transconjugants (Km^Rmutants).

2.13 Screening for mutants deficient in antibacterial activity

Using a sterile loop, transconjugant colonies were picked out from designated stock plates and inoculated into centrifuge tubes to yield sufficient aeration. The tubes contained 1 ml of MRS broth without antibiotic; in the mini-Tn*5*, the transposase (*Tnp*) is located outside the inverted repeats which prevents further transposition and ensures the stability of Tn*5* in target DNA (Dennis & Zylstra, 1998). Bacterial culture samples of mutant strains were incubated at 16^{o}C under shaking condition (120 rpm) for 72h. Screening was done by performing a spot agar assays using the mutants CFCS against the indicator strains *B. cereus* and (F). Mutant strains which lacked antibacterial activity in the CFCS, eg., lack of a zone of inhibition against the indicator strain were isolated and subjected to further tests.

2.14 DNA methods

All primers used in this study were purchased from MWG Eurofinns. Primers were re-suspended in sterile distilled water to a final concentration of (100 pmol/µl) before being stored at -20^{o}C.

Table 2.2 Classification of primers used in this study

Name	**Sequence (5′–3′)**	**Target**
AGPT-F	ATTCAACGGGAAACGTCTTG	Km^R gene[1]
AGPT-R	ACTGAATCCGGTGAGAATGG	Km^R gene[1]
tpnRL17-1	AACAAGCCAGGGATGTAACG	Outward directed primer[2]
tpnRL13-2	CAGCAACACCTTCTTCACGA	Outward-directed primer[2]

1. Callum Scott Thesis, Heriot Watt University

2. Larsen *et al*., 2002

2.14.1 Genomic DNA Extraction

Extraction of genomic DNA from bacterial strains was performed using the Thermo Scientific GeneJET Genomic DNA Purification Kit using the Gram-Negative Bacteria Genomic DNA Purification Protocol and following manufacturer's instructions. Gel electrophoresis was performed to evaluate the efficiency of the method. Genomic DNA samples were stored at -20^{o}C until needed.

2.14.2 Concentration of DNA (Ethanol precipitation)

Concentration of DNA (Genomic and plasmid) was performed using ethanol precipitation as follows: 1 volume of DNA was mixed with 1/10 volume of 3M Sodium Acetate (pH 5.2) and 2 volumes of ice cold Ethanol (96% vol/vol) before placing the sample at -20^{o}C for 1hr. The sample was centrifuged at 13000 rpm for 10mins and the supernatant was discarded.
The pellet was washed with 1 ml Ethanol (70%) and the sample was centrifuged as previously mentioned. The supernatant was discarded, and the pellet was air-dried at RT for 1 hr. The DNA was re-suspended in 20 µl of 1X TE buffer [Formula/ L: 10 ml of 10mM Tris-HCl (pH 7.5), 2 ml of 1mM EDTA and 988 ml of sterile distilled water] and stored at -20^{o}C until needed.

2.14.3 Gel electrophoresis

The quality of DNA samples was evaluated by performing agarose gel electrophoresis (Figure 2.6). A sartorius analytical balance was used to weigh the agarose powder. The agarose gels were prepared by dissolving 0.28 g of agarose powder in 30 ml of 1x TAE buffer (pH 8.0); [Formula / L: 50 x TAE buffer: Tris base; 242.0g, Glacial acetic acid; 57.1 ml, 0.5 M EDTA pH 8.0; 100ml] in a microwave oven. The melted gel was left to cool at RT for 5-10 mins before adding 1 µl of Ethidium Bromide (EB); (Concentration 0.5 µg/ml). The melted gel was poured into a gel tray containing an appropriate gel comb and was left to settle for 15mins. The comb was removed carefully and the gel tray was submerged with 1x TAE buffer. All samples including a DNA marker (Thermo scientific 1 Kb ladder; marker) were mixed with Thermo Scientific 6X DNA Loading Dye; according to manufacturers' instructions before being loaded into the gel wells and

run for 1 hr at 60 Volts. DNA fragments were visualized using BIO RAD Molecular Imager® ChemiDoc™ XRS+ system with image lab software.

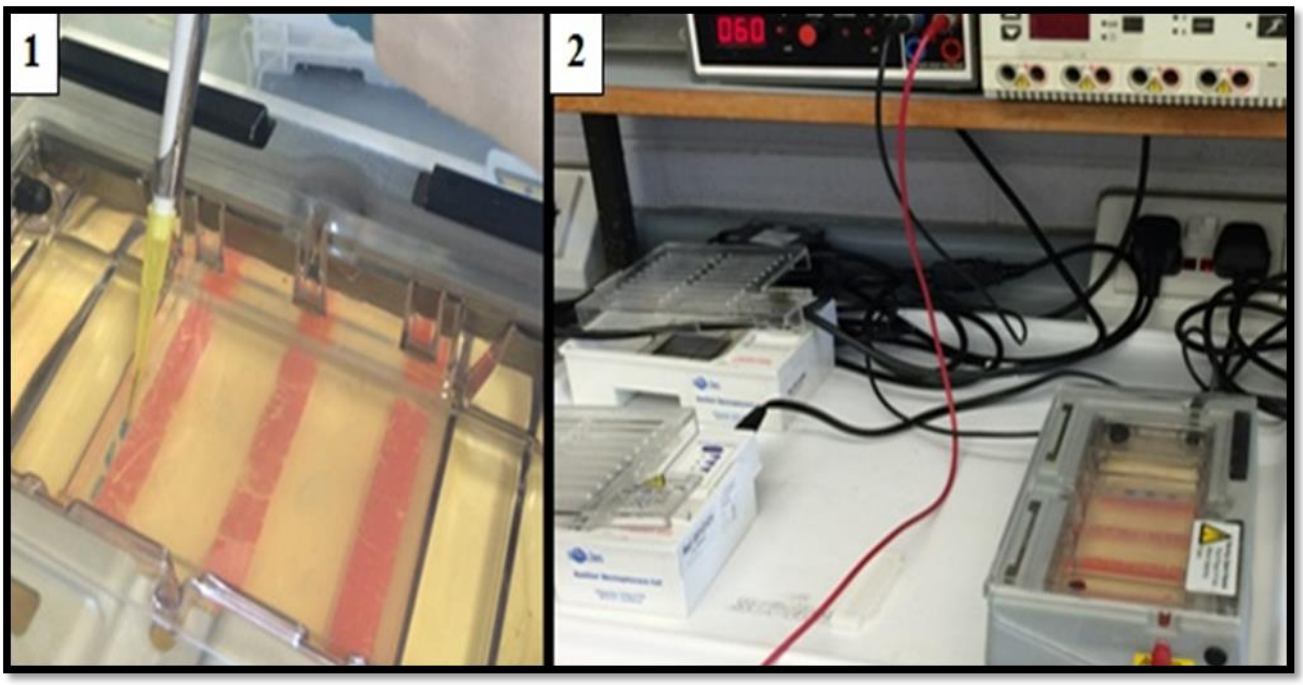

Figure 2.6: Demonstration of gel electrophoreses. 1; loading DNA samples into the gel wells, 2; an electric current is applied (60V/1hr) and DNA molecules are separated according to molecular size.

2.15 Polymerase Chain Reaction (PCR)

PCR was performed using Axygen® PCR tubes and contained the following: [DNA template 5µl, 10X Dream Taq buffer (1.25 ml) 5µl, dNTP mix (10mM) 0.5 µl, forward Primer (10 pmol/µl) 1 µl, reverse primer (10 pmol/µl) 1 µl, Dream Taq DNA Polymerase (5U/ µl,500U) 0.25 µl, Sterile deionized distilled water 37.25 µl]. PCR samples were placed in the GeneAmp® PCR System 2700 thermal cycler (Figure 2.7) and a standard PCR cycle thermal reaction was performed as shown in the following table. The PCR reaction mix was carried in a total volume of 50 µl.

Table 2.3 Thermal conditions for PCR reaction

Step	**Temperature**	**Time**
Denaturation	94ºC	2mins
25 Cycles	94 ºC	30sec
	53 ºC	30sec
	72 ºC	1min
Extension	72ºC	7mins
Hold	4ºC	∞

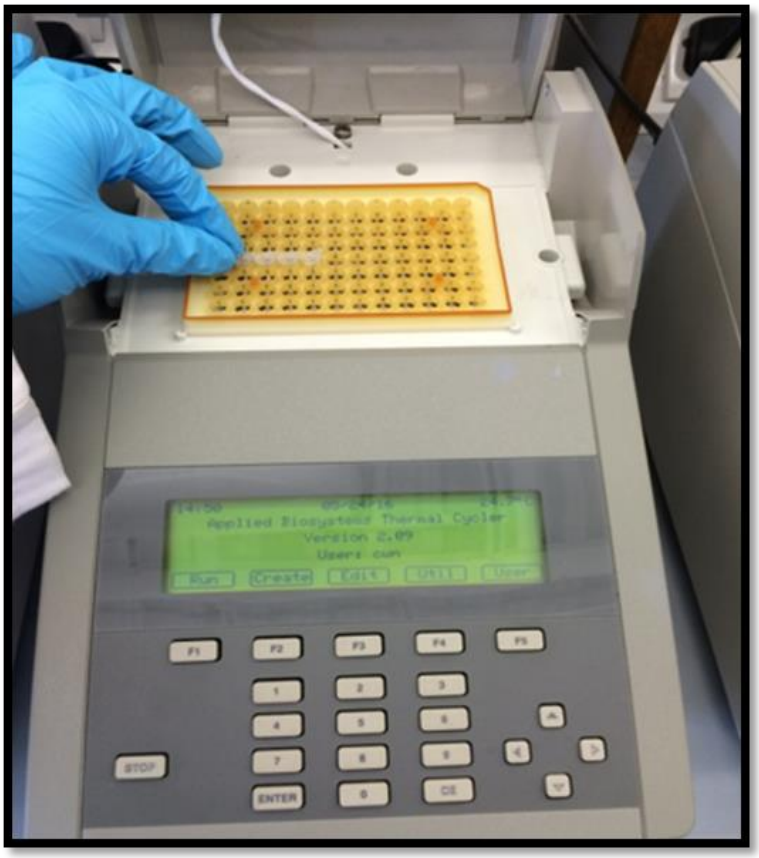

Figure 2.7: Demonstration of a PCR reaction performed in GeneAmp® PCR System 2700 thermal cycler.

2.16 Enzymatic digestion

Enzymatic digestion of DNA (Genomic DNA/plasmid DNA) was performed using the restriction enzymes *Bam*HI and *Eco*RI.

2.16.1 Enzymatic digestion using *Bam* HI

Enzymatic digestion was performed in 1.5 ml sterilized eppendorf tubes. The reaction mix contained: sterilized deionized distilled water (30 µl), 10X FastDigest Buffer (5 µl), DNA sample (Genomic/plasmid) (10 µl) and Thermo Scientific FastDigest *Bam*HI (5 µl). The samples were mixed for 30sec then incubated at 37°C for 5mins. The digestion reaction was terminated by heat inactivating the enzyme activity by placing samples in a hot water bath (80°C) for 5mins. DNA samples were left to cool at RT for 15mins before being stored at -20^{o}C until needed.

2.16.2 Enzymatic digestion using *Eco*RI

Enzymatic digestion was performed in 2 ml sterilized eppendorf tubes. The reaction mix contained the following: sterilized deionized distilled water (16 µl), 10X Buffer *Eco*RI (2 µl), DNA sample (Genomic/plasmid) (2 µl) and Thermo Scientific *Eco*RI (1 µl). The samples were mixed for 30sec and left to incubate at 37°C for 1hr. The digestion reaction was terminated by heat inactivation (20mins/65^{o}C). DNA samples were left to cool at RT before being stored at -20^{o}C until needed.

2.16.3 Concentration of digested DNA

Digested DNA samples were concentrated and purified using two methods: QIAGEN QIAXII gel extraction kit; (Desalting and concentrating DNA solutions protocol) and QIAquick PCR purification kit according to manufacturers' instructions.

2.17 DNA ligation

Ligation of DNA samples was performed by using Thermo Scientific T4 DNA Ligase (5 Weiss U/µL) and 10X T4 DNA Ligase Buffer and performed in 1.5 ml sterile eppendorf tubes. The ligation mix contained the following: digested DNA (1.5µl), sterilized deionized distilled water (25.5 µl). The sample was mixed gently by pipetting up and down 2-3 times before adding 10x T4 DNA ligase buffer (2 µl) and T4 DNA Ligase (1 µl). The samples were mixed for few sec followed by incubation at 16^{o}C for 24h. The ligation reaction was terminated by heat inactivation (65°C/10mins). All DNA samples were stored at -20^{o}C until needed.

2.18 Preparation of competent cells

2.18.1 Electro-competent *E. coli* cells

Single colonies from a fresh plate of *E. coli* (S17-1 λpir) were picked aseptically and inoculated into sterile 100 ml flask containing 20 ml of LB broth and incubated at 37^{o}C with shaking (120 rpm) for 24h. The overnight culture (1 ml) was inoculated into sterile 500 ml flasks containing 100 ml of pre-warmed LB broth and incubated at 37^{o}C with shaking (120 rpm) until OD_{600}~0.5-0.6. Bacterial culture samples were transferred to pre-chilled 50 ml centrifuge tubes and placed on ice for 5mins. Centrifugation was done at 4^{o}C for 5mins at 3000 rpm. The supernatant was discarded, and 50 ml of ice cold sterilized 50% glycerol was added to the cells and spun again at 4^{o}C and the supernatant was removed; (step repeated 4 times). The cells were re-suspended in 50% glycerol (500 µl) and divided into 100 µl aliquots and stored at -80^{o}C until needed.

2.18.2 Chemically competent *E. coli* cells

This method was prepared according to the protocol by Sambrook *et al*., (1989). The bacterial strain *E. coli* (S17-1 λpir) was cultivated in 20 ml of LB broth media at 37^{o}C with shaking (120 rpm) for 24h. The overnight culture (1 ml) was added to 100 ml of pre-warmed LB broth and incubated at 37^{o}C under shaking conditions (120 rpm) until OD_{600}~0.5-0.6. Bacterial culture samples were placed on ice for 15mins. Centrifugation was done at 4^{o}C for 10mins at 3000 rpm. The supernatant was discarded and 10 ml of ice cold 75mM $CaCl_2$ was added to the cells and placed on ice for 20mins followed by another centrifugation step. The supernatant was discarded and pre-chilled transformation buffer TFB2 (4 ml); (10 mM 3-(N-morpholino) propanesulfonic acid (MOPS) [pH 7.0], 75 mM $CaCl_2$, 10 mM RbCl and 15% Glycerol) was added to the cells. The cells were divided into 100 µl aliquots and stored at -80^{o}C until needed.

2.19 Cloning transposon insertion sites

2.19.1 Bacterial transformation

All centrifuge tubes, pipet tips and cuvettes were pre-chilled prior to transformation experiments by placing them at 4^{o}C for 24h in order to enhance transformation efficiency.

2.19.2 Transformation of competent cells by heat shock method

Frozen, chemically prepared, competent cells (100 µl) were thawed on ice before the addition of 10µl of the diluted ligation mixture (1:20). The sample was incubated on ice for 30mins followed by a heat shock reaction at (42°C/30 sec) then returned on ice for 5mins. Pre-warmed (37^{o}C) (1 ml) of SOC buffer [Contents per 200 ml; Yeast extract (1 g), Peptone (4 g), Glucose (0.712 g), NaCl 5M (400 µl), KCl 1M (500 µl), $MgCl_2$ 1M (2 ml), $MgSO_4$ 1M (4 ml)] was added to the sample. The sample was incubated at 37°C under shaking conditions (120 rpm) for 1 hr and centrifugation was done at 13,000 rpm for 1 min. This Incubation step allows the bacteria time to generate antibiotic resistance proteins encoded on the plasmid backbone, so they will be able to grow once plated on an antibiotic containing agar plate. The supernatant was discarded, and the pellet was re-suspended in 100 µl of room temperature SOC buffer. The sample (50 µl) was spread on pre-warmed LB plates + 50 µg/ml Km using sterile glass beads followed by incubation at 37°C for 24h.

2.19.3 Transformation of competent cells by electroporation

Electro-competent cells (100 µl) were thawed on ice. The diluted ligation mixture (1:20) was added to the cells (2µl) and the sample was gently mixed by pipetting up and down 2-3 times. The sample was transferred to a pre-chilled cuvette then placed into the Electroporator chamber of Eppendorf, Electroporator 2510. The sample was placed into the electroporator and an electric pulse (1700V) was applied. Immediately after electroporation, 950 µl of room temperature LB media was added to the sample and gently mixed by pipetting before being transferred to a 15-ml falcon tube and incubated at 37°C under shaking conditions (120 rpm) for 1hr; (cell recovery step that allows the

expression of antibiotic resistance). The sample (100 µl) was spread onto LB agar plates+(50 µg/ml Kanamycin) and incubated for 24h at 37°C.

2.20 Identification of transposon insertion sites

2.20.1 Purification of plasmid DNA

Single colonies of a fresh plate of the desired plasmid was picked aseptically and inoculated into 50 ml centrifuge tube containing 5 ml of LB broth+ (50 µg/ml) of the antibiotic kanamycin and incubated for 24h under shaking conditions (120 rpm) at 37ºC.
Purification was performed using Thermo Scientific GeneJET plasmid miniprep kit as described by the manufacturers' instructions.

2.20.2 DNA sequencing

DNA sequencing was performed using 8 strip tubes with a domed cap (Fisherbrand ™ 0.25 mL PCR tube strips). The sequencing reaction contained 6 µl and 1 µl of desired plasmid and primers respectively (Table 2.1 and 2.2). The samples were sent to Edinburgh Genomics and sequence results were compared with the databases in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the query BLASTX.

2.21 Concatenation of antibacterial activity

2.21.1 Rotary evaporation

Single colonies of the antibacterial producing strain were inoculated into 500 ml flasks containing 100 ml of the appropriate medium broth and incubated at 25ºC with shaking (120 rpm) for 48h /72h. The bacterial culture was distributed into 50 ml centrifuge tubes and centrifugation was performed at 3000x g for 15mins. The pellet was discarded and the culture supernatant was mixed with 100 ml of absolute ethanol and poured into the rotary evaporator (Heidolph) bulb. Temperature was set to 50˚C and pressure to 100 mbar (Figure 2.8).

Figure 2.8: Heidolph rotary evaporator used to concentrate respective samples by reducing the volume of the solutes.

2.21.2 Freeze-drying

Single colonies of the antibacterial producing strain were inoculated aseptically into 250 ml flasks containing 50 ml of NB broth and incubated at 25^{o}C with shaking (120 rpm) for 48h /72h. The bacterial culture was distributed into 50 ml centrifuge tubes and centrifugation was performed at 3000x g for 15mins. The supernatant was collected and placed into new centrifuge tubes and heat-sterilized at (70^{o}C/10mins). After cooling for 15mins at RT, the supernatant was distributed evenly in shallow plates in preparation for freeze drying. The plates were placed at -20^{o}C for 24h. Control sample; 50 ml of NB without bacterial culture. All samples were loaded into an Edwards Super Modulyo Freeze Dryer (Figure 2.9) for 48h.

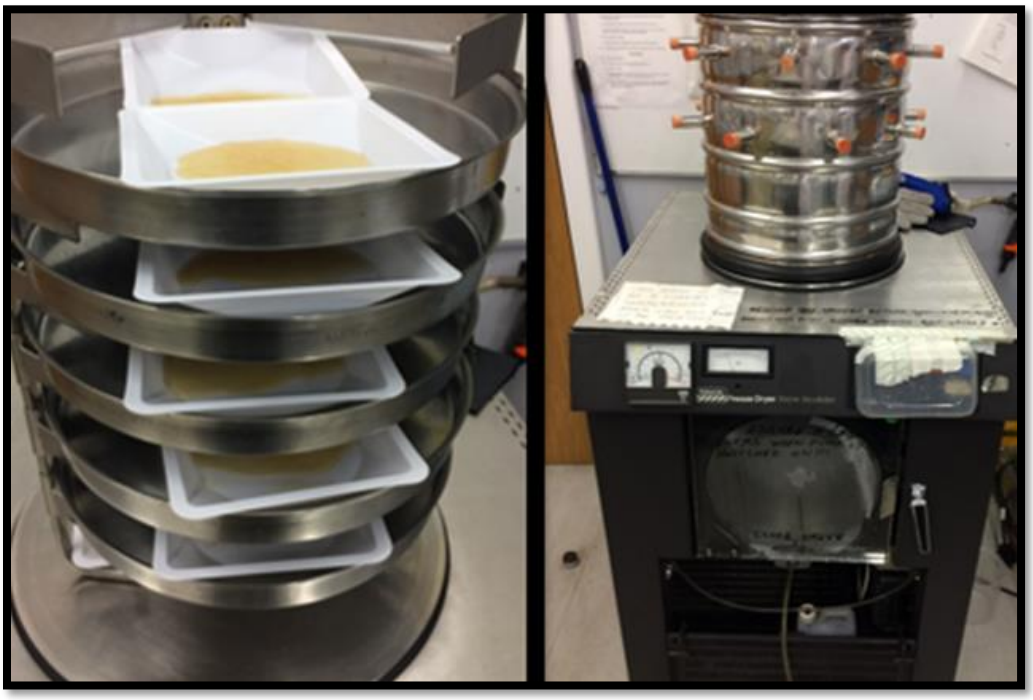

Figure 2.9: Edwards Super Modulyo Freeze Dryer used to concentrate respective samples. Freeze-drying is applied at (0.05 – 1) mbar and transforms solvents directly into vapour.

2.22 Purification of the antibacterial compound(s) by organic solvent extraction

The concentrated culture supernatant (CCS) was mixed with an equal volume of the organic solvent (methanol). Controls: (positive control; the CSS without the organic solvent, negative control; methanol only). All samples were incubated at 25^{o}C for 2h with shaking (120 rpm) and centrifugation was done at 3000xg for 15mins before uploading the samples into a vacuum concentrator (ThermoSavant AES2010 SpeedVac® System). Medium speed and heat conditions were applied. After evaporation, sterile deionized water (1 ml) was added to all samples before being stored at 4^{o}C until needed.

2.23 Nuclear magnetic resonance spectroscopy (NMR)

The desired samples were mixed with an equal volume of methanol. Control; methanol only. Samples were centrifuged at 3000xg for 15mins and uploaded into ThermoSavant AES2010 SpeedVac® System.

NMR was performed at the Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow. Respective samples were re-dissolved in 600 µl of Sigma-Aldrich Dimethyl Sulfoxide-d6 (DMSO-d6). For optimum dissolution, the samples were placed in a water bath for 3mins at 40^{o}C then transferred to Sigma-Aldrich 5mm NMR tubes. The samples were vortexed for few seconds. The NMR tubes were wiped off with a cloth before being placed in a spinner in order to eliminate any fingerprints which could interfere with the analysis. The NMR tubes were placed into a probe gauge and uploaded into the auto-sampler before being ready for analysis using JEOL AS400 NMR (Figure 2.10). NMR processing was done using the Mnova 10.0.1 software.

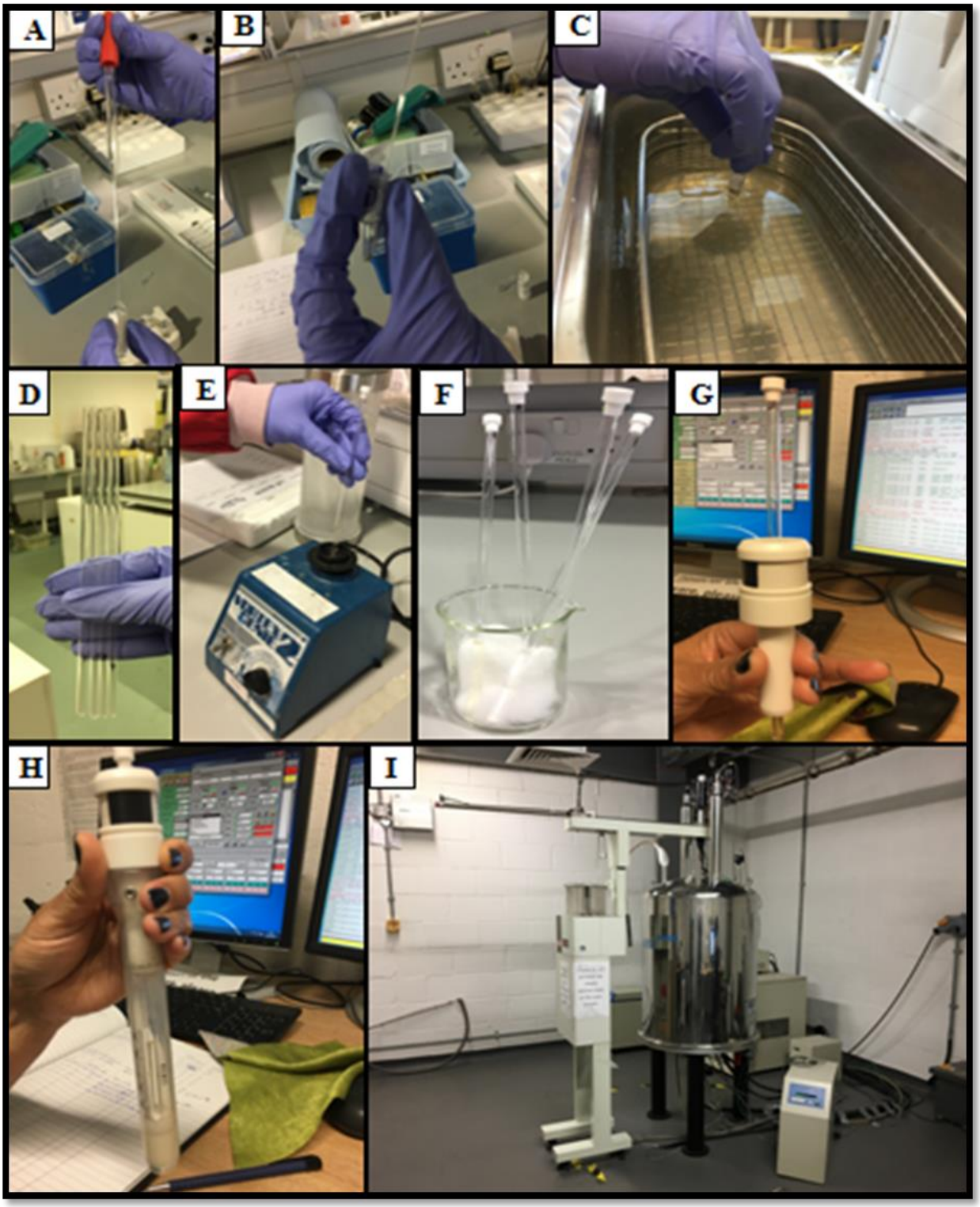

Figure 2.10: An overview of preparation steps leading to NMR spectroscopy. The respective samples were re-dissolved in 600 µl of DMSO-d6 and placed in a water bath (3mins at 40^{o}C). The samples were transferred to 5mm NMR tubes and mixed using a vortex for few seconds. The NMR tubes were placed into a probe gauge and uploaded into the auto-sampler before being ready for analysis using JEOL AS400 NMR.

CHAPTER 3: PROPERTIES OF THE SECRETED ANTIBACTERIAL ACTIVITY

3.1 Introduction

An environmental strain of *S. plymuthica* (Figure 3.1) isolated from the surface of the seaweed *Ascophyllum nodosum* has been shown to produce antibacterial activity against Gram-positive bacteria. This activity could be detected on agar plates after growing the microorganism aerobically in nutrient broth media NB for 18h at room temperature RT with shaking (120 rpm). The presence of inhibitory activity was assessed as previously mentioned in Section 2.5 against an appropriate target strain in this case; the Gram-positive bacterial strain (F) (Figure 3.2).

A previous study reported the isolation of *S. plymuthica* Km^R transposon mutants deficient in the activity detected on agar plates but possessed another activity secreted in liquid media. Those mutants had Tn*5* insertions into genes encoding polyketide synthases AlThubiani, (2013). The aim of this study is to investigate the production of the secreted antimicrobial activity in liquid media.

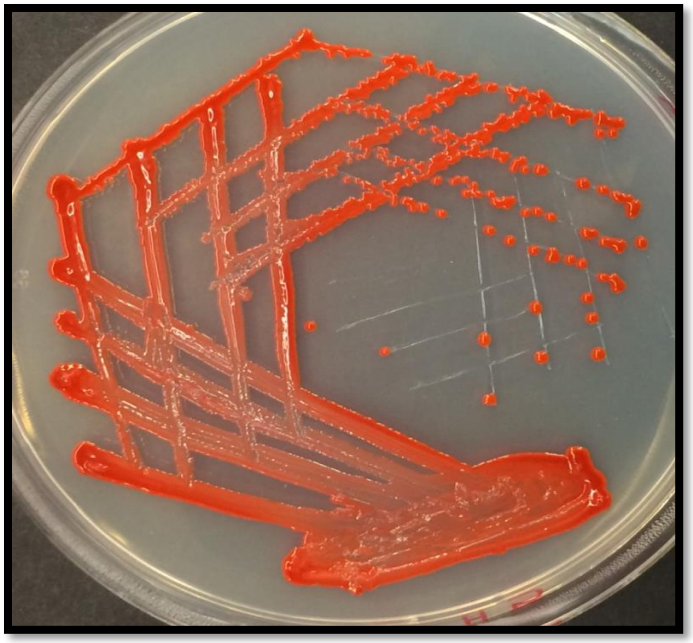

Figure 3.1: Colony morphology of the wild type strain *S. plymuthica* compromising small to medium mucoid orange shiny colonies on nutrient agar plate after growing for 18h at room temperature.

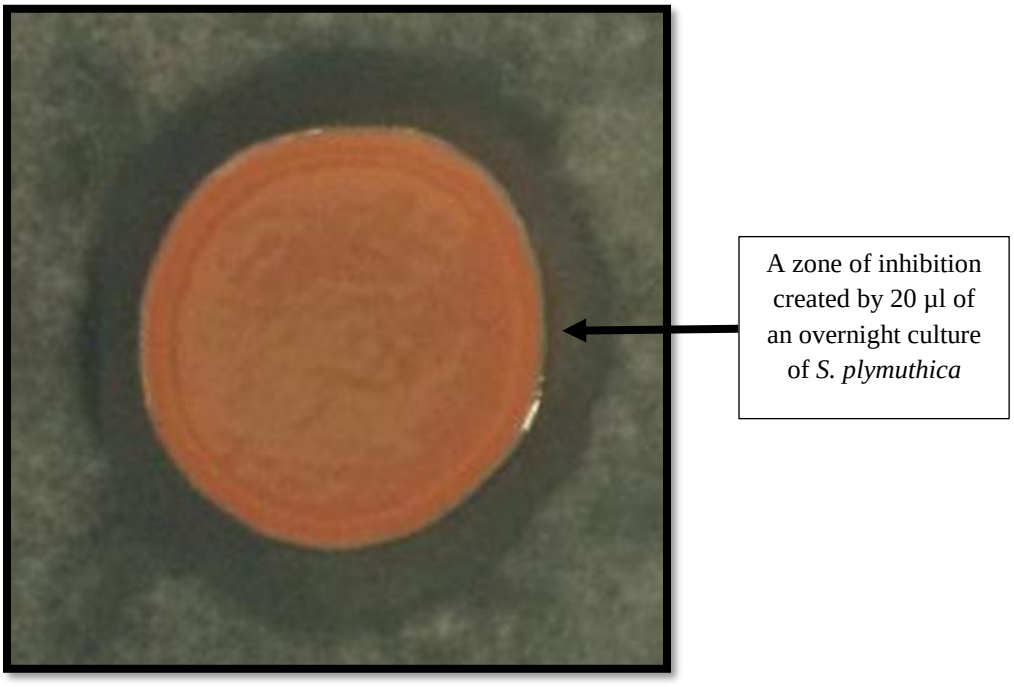

Figure 3.2: The antibacterial activity of the wild type strain *S. plymuthica* towards the Gram-positive strain (F); NA(RT/24h). This image is a representative image of 3 replicates.

3.2 Optimization of secreted antibacterial production

3.2.1 Preparation of cell-free culture supernatants (CFCS)

In order to investigate the secreted antibacterial activity of *S. plymuthica*, it was essential to prepare a cell-free culture supernatant CFCS that contained this activity. This was done by cultivating the antibacterial producing strain in 500 ml flasks with 100 ml of nutrient broth NB at 25^{o}C for 48h with shaking (120 rpm) followed by centrifugation (3000x g at 4^{o}C for 15mins). The culture supernatant of the antibacterial producing stain was collected and distributed into two groups.

The effect of two sterilization approaches was investigated in order to establish the best method to eliminate remaining *S. plymuthica* cells and prepare a CFCS that contains the desired antibacterial activity. The first group of the CFCS was sterilized by different heat-treatments (60^{o}C, 70^{o}C, 80^{o}C, 90^{o}C, 100^{o}C) for 10mins. While, the second group of the CFCS was filter-sterilized using a 0.2 µm cellulose acetate filter. The presence of activity was assessed against the Gram-positive strain (F).

Results showed that at 60^{o}C and 70^{o}C the CFCS produced activity against the Gram-positive strain with an inhibition zone diameter of 11.3 mm. While, at 80^{o}C and 90^{o}C produced an inhibition zone diameter of 11.0 mm. There was a slight decrease in activity at 100^{o}C with an inhibition zone diameter of 10.6 mm (Table 3.1). Results also showed that filter-sterilization does not inhibit or reduce the activity present in the CFCS (Figure 3.3). For the purpose of this study, heat-sterilization at 70^{o}C was used as the main temperature for sterilizing the culture supernatant.

The CFCS maintained its activity against the Gram-positive strain; the CFCS sample previously subjected to sterilization at (100^{o}C) was autoclaved (121^{o}C/15mins). The sample produced an inhibition zone diameter of 10.6 mm (Figure 3.4) which indicates that the secreted antibacterial is thermo-stable and tolerates autoclaving temperatures.

Table 3.1: Effect of heat-treatment on the secreted antibacterial activity. The activity is assessed against the Gram-positive strain (F). Inhibition zone sizes are in millimeters. Data shown are the means ± SD, n=3

Temperature	Inhibition zone
60^{o}C	11.3±0.57
70^{o}C	11.3±1.15
80^{o}C	11±1.0
90^{o}C	11±0
100^{o}C	10.6±0.57
121^{o}C	10.6±0.57

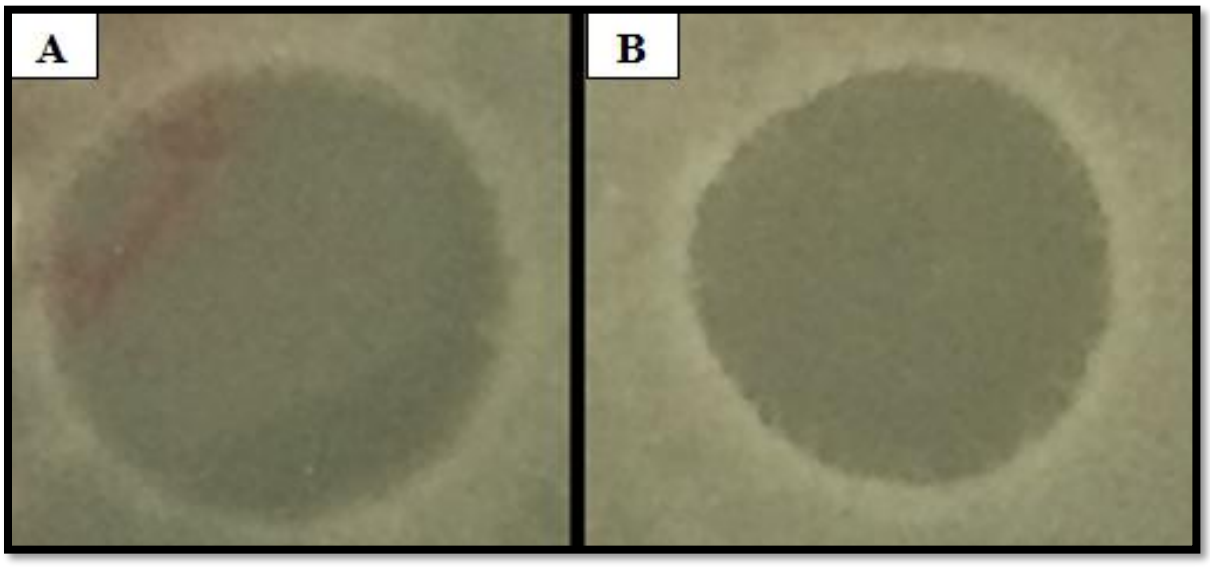

Figure 3.3: Demonstration of the inhibitory activity of *S. plymuthica* CFCS against the Gram-positive strain (F) ; NA (RT/24h). The strain was cultivated in NB broth at 25^{o}C for 48h with shaking (120 rpm).The bacterial culture was centrifuged (3000x g at 4^{o}C for 15mins).The supernatant was collected and sterlized via two methods. A; a zone of inhbtion (12.0 mm in diamter) obtained via filter sterilizing *S. plymuthica* culture supernatant through a 0.2 µm cellulose acetate filter. B; a zone of inhbtion (11.0 mm in diamter) obtained via heat sterlizion of *S. plymuthica* culture supernatant. This image is a representative image of three replicates.

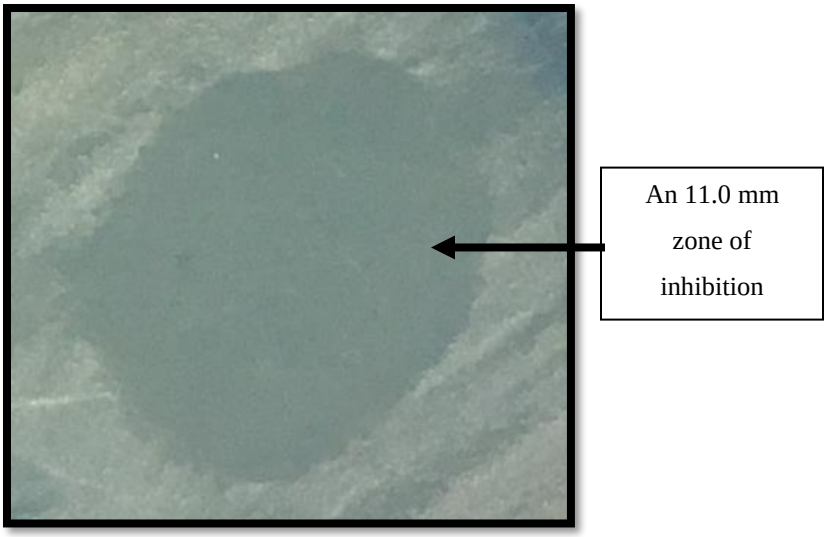

Figure 3.4: The thermo-stability of the secreted antibacterial activity. The CFCS of the antibacterial producing strain; *S. plymuthica* was autoclaved (121ºC/15mins) and the presence of activity was assessed against the Gram-positive strain (F); NA(RT/24h). This image is a representative image of 3 replicates.

3.2.2 Influence of incubation period on the production of secreted antibacterial activity

The influence of incubation period on the production and amount of inhibitory activity detected in the CFCS was investigated in order to establish the optimum incubation period for the production of antibacterial activity in *S. plymuthica* culture supernatant. The antibacterial producing strain was incubated at the following incubation periods: 24h, 48h, 72h, 96h and 120h and the presence of activity was assessed against (F). Results showed that the inhibitory activity was produced after 24h of cultivation and the CFCS produced an inhibition zone diameter of 8.3 mm against the Gram-positive strain. The highest amount of antibacterial activity detected in the CFCS was after 48h and 72h of cultivation with inhibition zones diameter of 8.6 and 9.0 mm respectively. However, the amount of inhibitory activity in the CFCS was decreased after a cultivation period of 96h and 120h with inhibition zones diameter of 8.3 and 7.0 mm respectively (Table 3.2). These results indicate that (48-72h) is the optimum incubation period for the production of secreted antibacterial activity detected in the CFCS; which could be the stationary phase for the antibacterial producing strain.

Table 3.2: The influence of incubation period on the production of secreted antibacterial activity. The activity is assessed against the Gram-positive strain (F). Inhibition zone sizes are in millimeters. Data shown are the means ± SD, n=3

Incubation period (hours)	**Inhibition zone**
24h	8.3±0.33
48h	8.6±0.33
72h	9.0±0
96h	8.3±0.33
120h	7.0±0

3.2.3 Influence of cultivation temperatures and media composition on the production of secreted antibacterial activity

In order to establish the optimum medium for the production of the secreted antibacterial activity. The antimicrobial producing strain *S. plymuthica* was inoculated in 250 ml sterilized flasks containing 20 ml of different broth media. Those included: NB, LB, BHI and MRS. Bacterial cultures were incubated aerobically across the following temperatures (16^{o}C, RT, 30^{o}C, 35^{o}C) for 72h with shaking 120 rpm. Bacterial cultures were centrifuged at (3000x g/ 15mins) and the supernatants were collected followed by heat-sterilization (70^{o}C/10mins). The presence of activity was assessed against the following Gram-positive strains: *S. aureus*, *B. cereus*, *B. subtilis* and (F). Control broth media without bacterial cells were similarly tested.

3.2.3.1 The influence of media composition at 16ºC on the production of secreted antibacterial activity

At 16ºC, all CFCS obtained from broth media produced inhibitory activity against the Gram-positive strains (Table 3.3). The best broth media for the production of antibacterial activity detected in the CFCS was MRS, followed by BHI, NB then LB. The CFCS obtained from MRS broth produced the highest amount of inhibitory activity and showed inhibition zones diameter of 14.3, 16.3, 17.3 and 12.6 mm against *S. aureus*, *B. cereus*, *B. subtilis* and (F) respectively.

The activity of the CFCS obtained from BHI broth produced activity against *B. subtilis* and (F) with inhibition zones diameter of 15.3 and 10.3 mm respectively. The CFCS obtained from BHI broth produced intermediate activity against *S. aureus* and *B. cereus* with inhibition zones diameter of 11.0 mm.

The CFCS obtained from NB broth showed intermediate inhibitory activity against *B. subtilis* and (F) with inhibition zones diameter of 11.6 and 8.3 mm respectively. The lowest activity detected in the CFCS from NB broth was against *S. aureus* and *B. cereus* with inhibition zones diameter of 3.3 and 6.0 mm respectively.

LB broth was the least suitable medium for the production of inhibitory activity. The CFCS obtained from LB broth produced intermediate activity against *B. subtilis* and (F) with inhibition zones diameter of 10.3 and 9.0 mm. The lowest activity detected in the CFCS from LB was against *S. aureus* and *B. cereus* with inhibition zones diameter of 3.6 and 3.3 mm respectively. These results indicate that at 16ºC, MRS and BHI are the optimum broth media for the production of inhibitory activity detected in the CFCS. Results also showed that at 16ºC, *B. cereus* and *B. subtilis* are the most sensitive strains to the inhibitory activity.

Table 3.3: The influence of media composition at 16°C on the production of secreted antibacterial activity. The activity is assessed against indicator Gram-positive bacteria. Inhibition zone sizes are in millimeters. Data shown are the means ± SD, n=3

	Inhibition zone			
Media broth	***S. aureus***	***B. cereus***	***B. subtilis***	**(F)**
LB	3.6±6.4	3.3±5.8	10.3±1.2	9.0±1.7
NB	3.3±5.8	6.0±5.2	11.6±2.1	8.3±1.5
BHI	11.0±2.0	11.0±1.0	15.3±4.5	10.3±2.5
MRS	14.3±1.5	16.3±3.5	17.3±4.6	12.6±2.3

3.2.3.2 The influence of media composition at RT on the production of secreted antibacterial activity

At room temperature (RT), not all CFCS obtained from broth media produced inhibitory activity against the Gram-positive indicator strains (Table 3.4). CFCS obtained from LB broth media only produced activity against *B. subtilis* with an inhibition zone diameter of 10.3 mm. The lowest recorded activity was by the CFCS from NB broth with inhibition zones diameter of 6.6 and 3.3 mm against *B. subtilis* and *B. cereus* respectively and an inhibition zone diameter of 3.0 mm against (F) and *S. aureus*. The CFCS obtained from BHI broth produced intermediate activity against *B. subtilis* and (F) with inhibition zones diameter of 11.3 and 9.3 mm respectively while, produced low activity against *B. cereus* and *S. aureus* with inhibition zones diameter of 7.0 and 3.6 mm respectively. The CFCS obtained from MRS broth produced the highest amount of inhibitory activity with inhibition zones diameter of 12.6, 10.6, 11.0 and 10.0 mm against *S. aureus*, *B. cereus*, *B. subtilis* and (F) respectively. These results indicate that at RT, LB broth is the least suitable media for the production of antibacterial activity detected in the CFCS. The best broth media for the production of antibacterial activity detected in the CFCS is M.R.S and BHI followed

by NB broth. Results also showed that at RT, *S. aureus* and *B. subtilis* are the most sensitive strains to the inhibitory activity of the CFCS.

3.2.3.3 The influence of media composition at 30ºC and 35ºC on the production of secreted antibacterial activity

At 30ºC, the only recorded activity was from the CFCS obtained from MRS broth media with inhibition zones diameter of 6.6, 9.0, 10.3 and 10.6 mm against *B. cereus*, (F), *B. subtilis* and *S. aureus* respectively (Table 3.5). Also, at 35ºC, there was no presence of inhibitory activity in the CFCS obtained from any of the broth media against the Gram-positive indicator strains.

Table 3.4: The influence of media composition at RT on the production of secreted antibacterial activity. The activity is assessed against indicator Gram-positive bacteria. Inhibition zone sizes are in millimeters Data shown are the means ± SD, n=3

	Inhibition zone			
Media broth	***S. aureus***	***B. cereus***	***B. subtilis***	**(F)**
LB	0	0	10.3±1.5	0
NB	3±5.2	3.3±5.8	6.6±5.8	3±5.2
BHI	3.6±6.4	7±6.1	11.3±0.6	9.3±1.5
MRS	12.6±1.5	10.6±1.2	11.0±1.0	10±1.0

Table 3.5: The influence of media composition at 30°C on the production of secreted antibacterial activity. The activity is assessed against indicator Gram-positive bacteria. Inhibition zone sizes are in millimeters. Data shown are the means ± SD, n=3

	Inhibition zone			
Media broth	***S. aureus***	***B. cereus***	***B. subtilis***	**(F)**
LB	0	0	0	0
NB	0	0	0	0
BHI	0	0	0	0
MRS	10.6±1.2	6.6±5.9	10.3±2.1	9.0±0

3.2.4 Influence of different growth environments on secreted antibacterial production

In order to establish the best growth environment for the production of secreted antibacterial activity detected in the CFCS. The antibacterial producing strain *S. plymuthica* was cultured under different conditions; (96 well-plates, centrifuge tubes and glass flasks). Incubation was done aerobically at room temperature for 72h with shaking (120 rpm). The strain was inoculated in MRS broth since it was established that it is the optimum growth medium for the production of antibacterial activity (Section 3.2.3) and the presence of antibacterial activity was assessed against the Gram-positive strain (F). Results showed that growing the antimicrobial producing strain in 96 well-plates which contain ~300 µl of broth medium produced inconsistent results; making it very difficult to detect zones of inhibition. Likewise, growing the antibacterial producing strain in centrifuge tubes (50 ml) containing 5 ml of MRS broth also produced inconsistent results and in many cases lack of inhibitory activity. The optimum growth environment for the production of secreted antibacterial activity was 250 ml flasks containing 20 ml of MRS broth medium. The CFCS produced inhibition zones diameter of 10±1 (Table 3.6). These results indicate that the production of antibacterial activity is influenced by growth conditions and aeration availability.

Table 3.6: The influence of different growth environments on the production of secreted antibacterial activity. The activity is assessed against (F). (±) inconclusive results, (+++) strong activity. N=3

Growth environment	Presence of inhibitory activity
96 well- plates	±
Centrifuge tubes (50 ml)	±
Glass flasks (250 ml)	+++

3.3 Characterization of the secreted antibacterial activity

3.3.1 Effect of storage on the stability of secreted antibacterial activity

The effect of storage on the stability of secreted antibacterial activity was studied by preserving the CFCS at 4^{o}C for 7 days and recording the presence of activity against the Gram-positive indicator strain (F). Results showed that the inhibitory activity of the CFCS is affected by storage and decreases over time. The inhibition zones diameter decreased from 12.3 mm at day zero to 7.6 mm at day 7 as shown in the following table:

Table 3.7: The effect of storage at 4^{o}C on the stability of secreted antibacterial activity. The activity is assessed against the Gram-positive strain (F). Inhibition zone sizes are in millimeters. Data shown are the mean ± SD, n=3

Day	inhibition zone
Zero	12.3 ± 1.5
Day 5	11.6 ± 0.5
Day 7	7.6 ± 6.6

3.3.2 Effect of Proteinase K and TWEEN® 20 on the stability of the secreted antibacterial activity

The effect of Proteinase K on the stability of the secreted antibacterial activity was investigated in order to give an insight to the nature of the activity in the CFCS. The CFCS (100 µl) was mixed with (80 µl) of Proteinase K stock solution (20 mg/ml) and sterile distilled water (20µl) and incubated for 3h at 37ºC and as a control an identical volume of a diluted CFCS was incubated without Proteinase K. The presence of activity was assessed against (F). Results showed that both the CFCS and the control sample produced zones of inhibition against the Gram-positive strain with inhibition zones diameter of 14.3±3.7 and 15±3.6 respectively (Figure 3.5). These results indicate that the antibacterial activity of the CFCS is resistant to Proteinase K and unlikely to be a protein.

Regarding investigating the effect of the detergent TWEEN® 20 on the stability of the secreted antibacterial activity. This was done as follows: The CFCS was mixed with an equal volume of the detergent 1 % (v/v). Controls; positive control: CFCS without TWEEN® 20 and negative control: TWEEN® 20 only. All samples were incubated at 37ºC for 3h and the presence of activity was assessed against the Gram-positive strain (F). Results showed that the inhibitory activity of the CFCS was not affected by the detergent. The CFCS produced activity with an inhibition zone diameter of 14.5 mm. The positive control (CFCS without detergent) produced an inhibition zone diameter of 15.0 mm (data not shown) while, the negative control (TWEEN® 20 only) showed no signs of inhibitory activity (Figure 3.6).

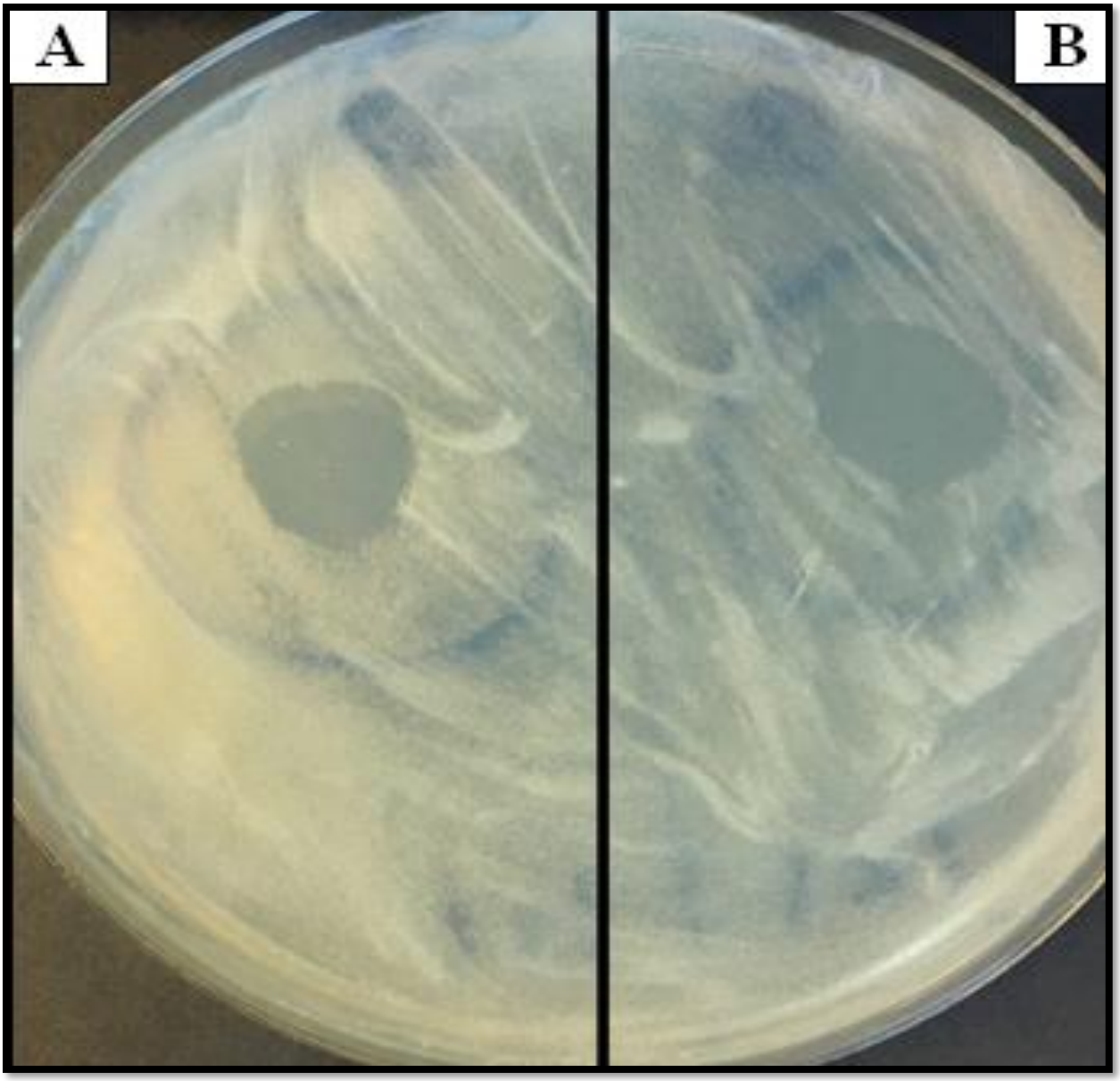

Figure 3.5: The effect of Proteinase K on the stability of secreted antibacterial activity. The CFCS was incubated with Proteinase K for 3h at 37ºC. The presence of activity was assessed against the Gram-positive strain (F); NA (RT/24h). A; zone of inhibition (19.0 mm) produced by the control (CFCS without Proteinase K). B; zone of inhibition (17.0 mm) produced by the (CFCS + proteinase K). This image is a representative image of 3 replicates.

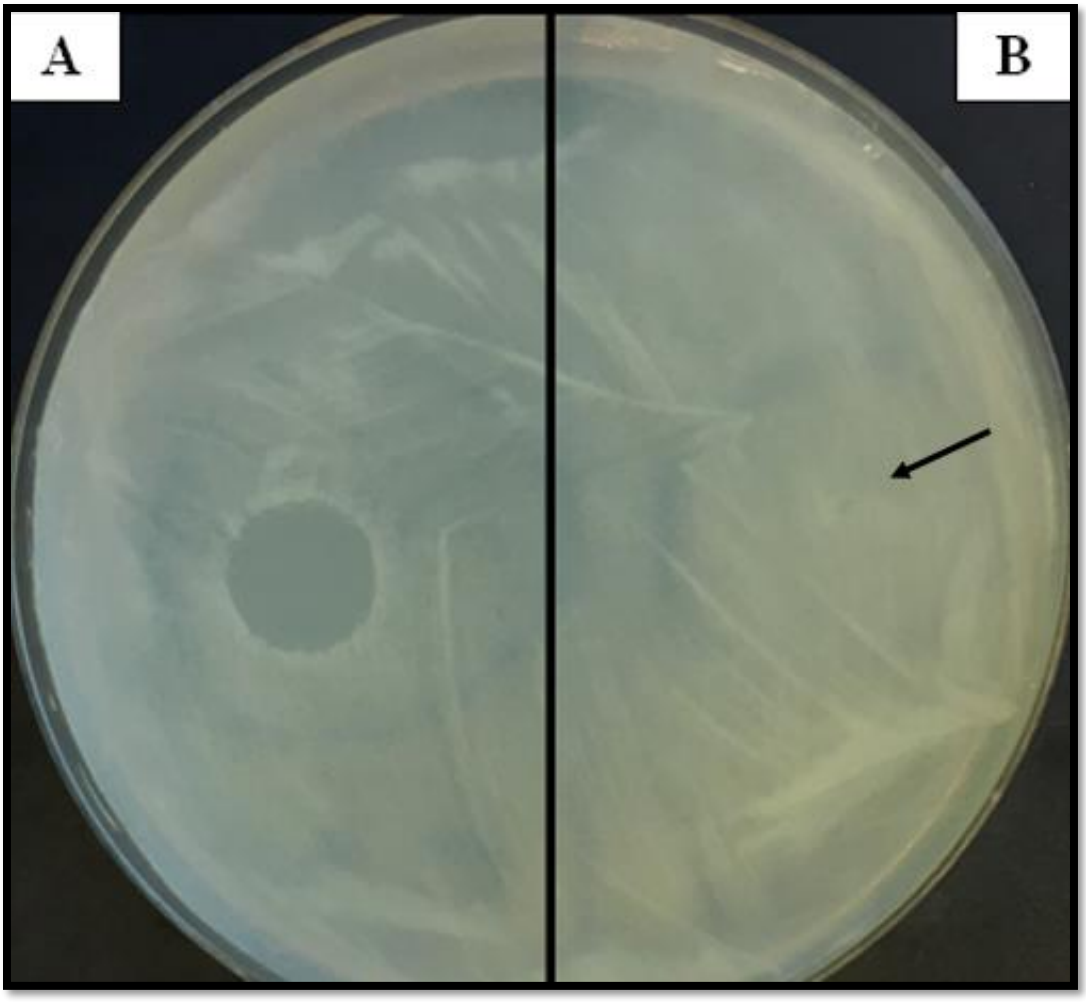

Figure 3.6: The effect of the detergent (TWEEN® 20) on the stability of secreted antibacterial activity. The CFCS was mixed with an equal volume of the detergent and incubated for 3h at 37ºC. Negative Control; (detergent only). The presence of activity was assessed against the Gram-positive strain (F); NA (RT/24h). A; zone of inhibition (14.0 mm) produced by the CFCS after incubation with TWEEN® 20, B; lack of inhibitory activity of the control (TWEEN® 20). This image is a representative image of three replicates.

3.3.3 Effect of pH on the stability of the secreted antibacterial activity

The effect of pH on the stability of secreted antibacterial activity detected in the CFCS was investigated as follows: A fresh sample of the CFCS (30 ml) was distributed into 3 groups and named A, B and C. The pH of the CFCS was measured (pH 7.3±0.2) and recorded as initial pH. Group A was subjected to acidic conditions by adjusting the pH to 5 using HCl (1M). Group B was subjected to alkaline conditions by increasing the pH to 9 using NaOH (1M). Group C was the control sample (The CFCS without any changes to the pH). All samples were incubated at 22^{o}C for 2h with shaking (120 rpm). The pH of samples (A) and (B) were returned to the initial pH using HCl and NaOH. The presence of activity in all samples was assessed against the Gram-positive strain (F). Results showed that all samples produced activity against the indicator strain (Table 3.8). The CFCS produced activity with inhibition zones diameter of 11.0 mm at pH 5 and 10.0 mm at pH 9. The positive control; (CFCS without pH changing) produced activity with an inhibition zone diameter of 13.3 mm in diameter. These results indicate that the antibacterial activity is affected by changing the pH of the CFCS.

Table 3.8: The effect of pH on the stability of secreted antibacterial activity. The activity is assessed against the Gram-positive strain (F); NA (RT/24h). Inhibition zone sizes are in millimeters. Data shown are the mean ± SD, n=3

pH	**Inhibition zone**
pH 5	11 ± 0
pH 7.3 ± 0.2	13.3 ± 0.57
pH 9	10 ± 0

3.3.4 Effect of Tris buffer on the stability of secreted antibacterial activity

Tris-buffer was initially chosen to maintain the secreted antibacterial activity since it was established that the activity decreases over time at 4^{o}C (Section 3.3.1). The secreted antibacterial activity of the concentrated culture supernatant CCS (Section 5.1) was investigated in order to establish if the activity is affected by Tris-buffer pH 7.0; (50 mM), NaCl (50 mM), Glycerol (5%) and EDTA (2 mM).

The activity of the CCS was initially assessed against the Gram-positive strain (F) prior to incubation with Tris-buffer. The CCS produced inhibition zones diameter of 14.3±4.9 mm. The Tris-buffer was added to the CCS (10:1; Tris-buffer: sample) and incubated for 2h at 21^{o}C with shaking 120 rpm followed by centrifugation for 15mins. The supernatants (soluble fractions) were collected and placed into new centrifuge tubes and the precipitates were re-suspended in 1 ml of sterile deionized water.

The presence of activity in all samples was assessed as mentioned above. The CCS sample (soluble and precipitates fractions) produced activity against the Gram–positive strain with inhibition zones diameter of 10.0±0 and 10.3±0.6 mm respectively (Figure 3.7). These results indicate that the secreted bacterial activity was affected by the buffer making it an unsuitable choice to be used as a preservative. Also, the observed precipitate might be due to the presence of specific *Serratia* enzymes that metabolize organic acids and glycerol causing a precipitation reaction.

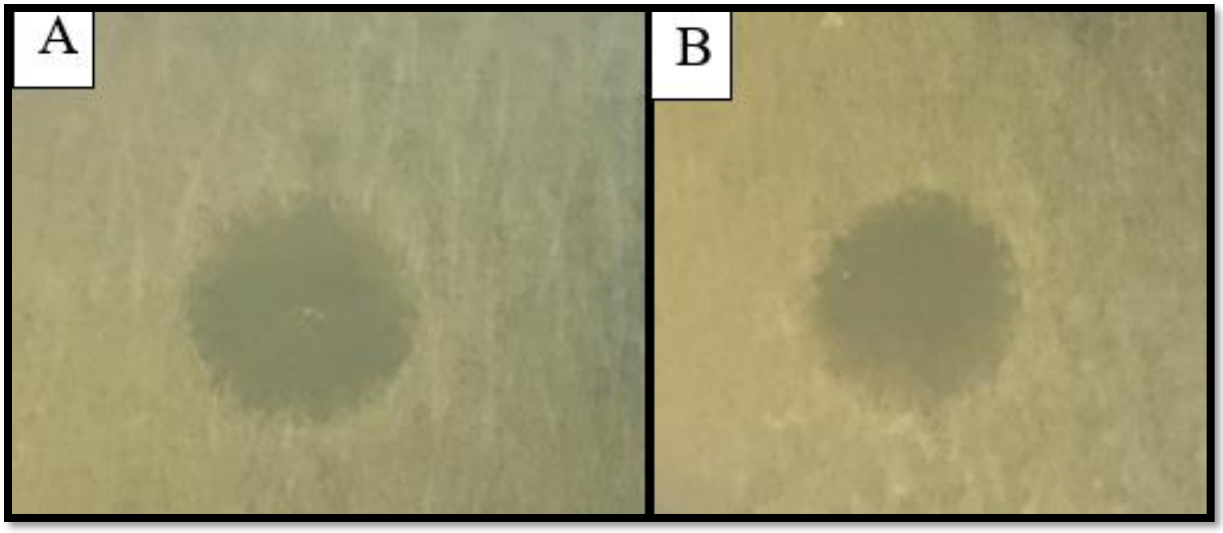

Figure 3.7: The Effect of Tris-buffer on the stability of secreted antibacterial activity. A Tris-buffer was incubated with the CCS (10:1) followed by incubation at 21^{o}C for 2h with shaking (120 rpm) then centrifugation for 15mins. The supernatant (Soluble fraction) was collected and the precipitate was re-suspended in 1 ml of sterile deionized water. The presence of activity was assessed against the Gram-positive strain (F). A; zone of inhibition (10.0 mm) produced by the soluble fraction. B; zone of inhibition (11.0 mm) produced by the precipitate fraction. This image is a representative image of 3 replicates.

3.3.5 Estimation of the molecular weight of the secreted antibacterial compound(s)

An ultra-filtration technique was used to estimate the size of the secreted antimicrobial compound(s) present in the culture supernatant of the antibacterial producing strain *S. plymuthica*. A centrifugal device with a 10,000 Dalton molecular weight cut-off membrane (MWCO) was used and centrifugation conditions were applied appropriately according to manufacturer's instructions. Centrifugation produced two fractions, a retained fraction and a flow through fraction; which for the purpose of this experiment were named Fractions A and B respectively. The presence of activity in the fractions was assessed against (F). Both fractions A and B produced activity against the Gram-positive strain with inhibition zones diameter of 15.0 mm±1.0 and 10.6 mm±9.3 respectively. These results might indicate the presence of two secreted antimicrobial compounds in the CFCS. A compound with a molecular weight ≥ 10,000 Daltons and a compound with a molecular weight < 10.000 Daltons.

In order to have an approximate size of the secreted antibacterial compound(s), another centrifugal device with a lower molecular cut-off was used (3000 MWCO) (Figure 3.8). Centrifugation also resulted in the production of two fractions. The retained fraction was named (Fraction C) while the flow through was named (Fraction D). The presence of activity was assessed against (F). Results showed that while Fraction (C) produced inhibitory activity with an inhibition zone diameter of 14.3±1.5 mm, (Fraction D) did not show any signs of activity (Figure 3.9). These results indicate the presence of two secreted antibacterial compounds in the CFCS, a compound with a molecular weight ≥10,000 Daltons and a compound with a molecular weight >3000 and <10,000 Daltons. Also, it is possible that there is only one antibacterial compound with portion forming larger aggregates.

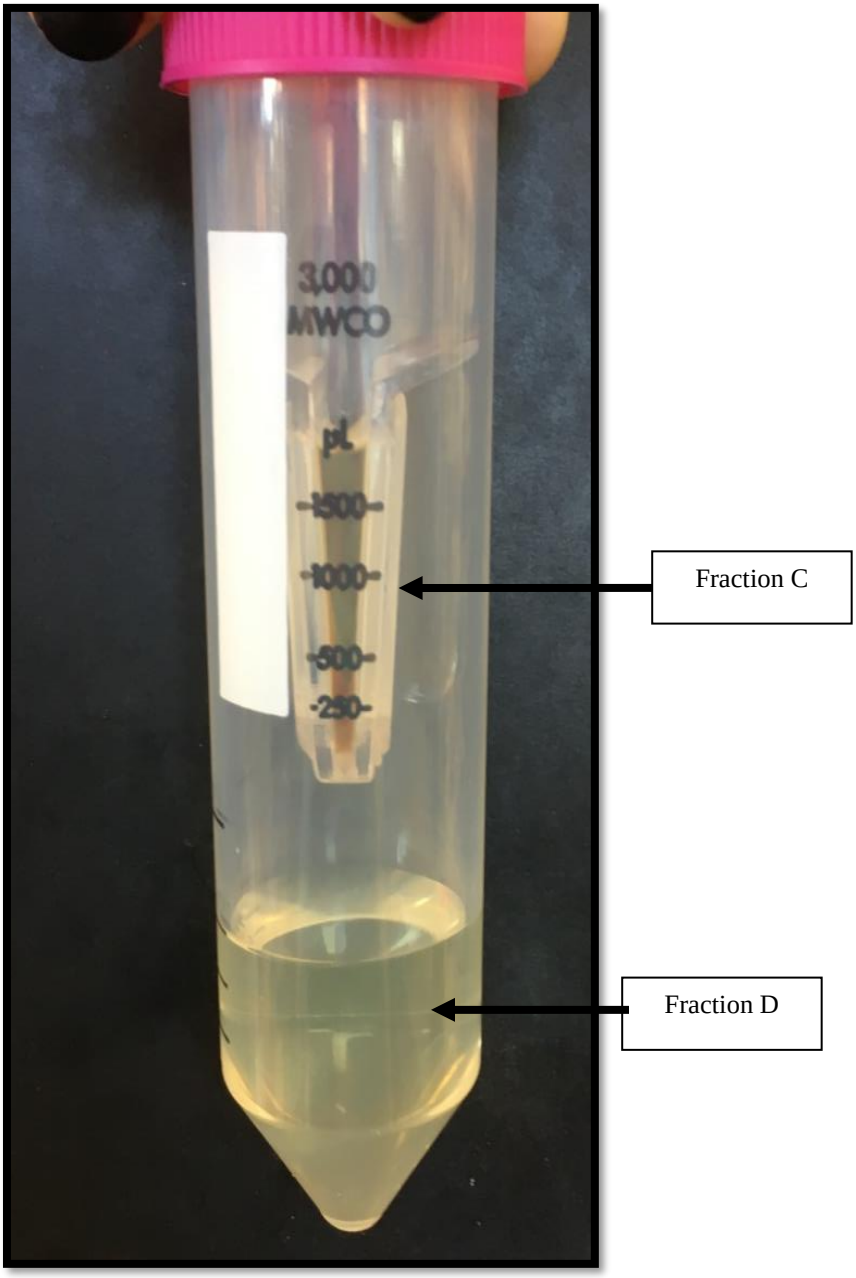

Figure 3.8: A centrifugal filter with a molecular weight exclusion of 3000 Daltons was used in this study to estimate the size of the secreted antibacterial compound (s) present in the CFCS. The presence of activity in both fractions was assessed against the Gram-positive strain (F); NA(RT/24h). Fraction C produced inhibitory activity against the Gram-positive strain however, fraction D did not show any signs of activity which indicates that the size of the secreted antibacterial compound(s) present in the CFCS is > 3000 Daltons.

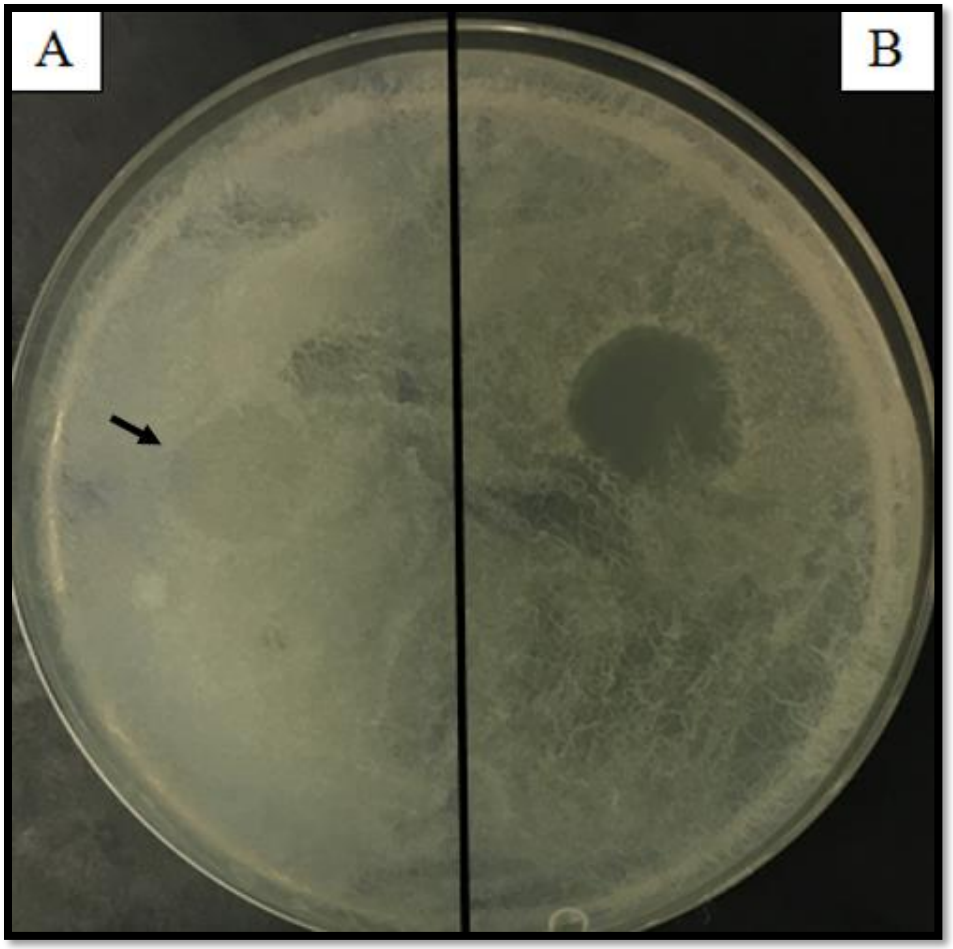

Figure 3.9: The activity of the CFCS retained (fraction C) and the flow through fraction (Fraction D) obtained via ultra-filtration using a centrifugal filter with a molecular weight exclusion of 3000 Daltons against the indicator strain (F); NA (RT/24h). A; lack of inhibitory activity in the flow through fraction, B; zone of inhibition (16.0 mm) produced by the retained fraction. This image is a representative image of 3 replicates.

3.3.6 Measuring the effect of antibacterial activity on bacterial growth

The biological nature of the secreted antibacterial activity of the CFCS was investigated to determine whether it has a bacteriostatic or a bactericidal effect. This was done as previously mentioned in Section 2.11.2 followed by the addition of different volumes of the CFCS (20,60,180, 540 and 1620 µl) to the cells of the Gram-positive strain *B. cereus*. All samples were incubated at 30ºC for 2h. As a control; (the cells of *B. cereus* were equally treated but without the CFCS). A series of 10-fold serial dilutions using sterile MRD were prepared from the samples and 100 µl of each dilution was plated onto nutrient agar plates and incubated at room temperature for 24h. Results are displayed in Figure 3.10 and showing the bactericidal activity of the CFCS; the larger the volume of CFCS added to the cells the lower the number of surviving colonies.

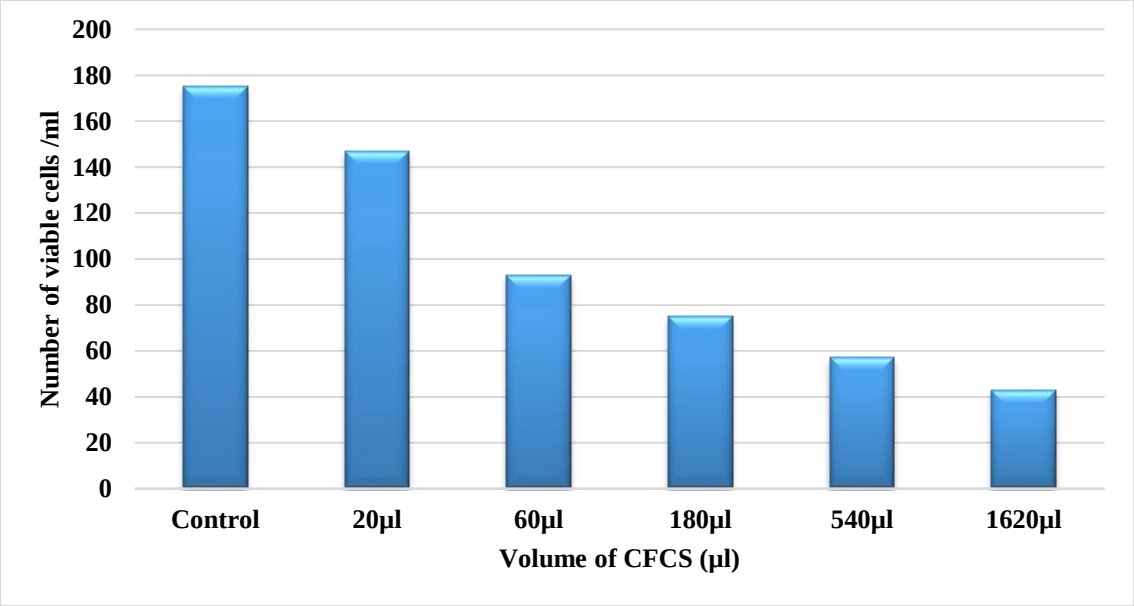

Figure 3.10: The bactericidal activity of the secreted antibacterial compound(s) present in the CFCS against the Gram-positive strain *B. cereus*. Different volumes of the CFCS were added to the cells of *B. cereus*. Control (*B. cereus* cells without CFCS). Serial dilutions (10 Fold Dilutions) were prepared and 100 µl of each dilution was plated onto NA plates and incubated at (RT/24h). Data represents the means. N=2.

3.3.7 Spectrum of secreted antibacterial activity

The CFCS was investigated to study the spectrum of antibacterial activity and to establish wither it is restricted to Gram-positive bacteria. The activity of the CFCS was tested against the following strains previously mentioned in Table 2.1. These included, *P. aeruginosa, K. pneumonia, S. marcescens, E. coli, C. tropicalis, S. diastaticus, C. krusei, S. cerevisiae* and *E. faecalis* and (S). The following Gram-positive bacteria *S. aureus, B. cereus, B. subtilis* and (F) were used as controls. Spot agar assays were performed using NA plates for bacteria and YPD plates for the yeast and fungi and incubated appropriately.

Results showed that the CFCS did not show any activity against any of the Gram-negative bacteria or fungal and yeast strains. Surprisingly, the CFCS also did not show any activity against the Gram-positive strain *E. faecalis*. The spectrum of the secreted antibacterial activity present in the CFCS is demonstrated in Table 3.9. These results indicate that the secreted antibacterial activity is species-specific targeting Gram-positive bacteria in particular those belonging to *Bacillaceae* confirming the results previously mentioned in Section 3.2.3.

Table 3.9: The spectrum of inhibitory activity of the secreted antibacterial compound(s) (+++) strong inhibitory activity, (-) no inhibitory activity. N=3

Indicator strain	**Inhibitory activity**
(F)	+++
Pseudomonas aeruginosa	-
B. cereus	+++
S. aureus	+++
B. subtilis	+++
(S)	-
Klebsiella pneumoniae	-
Enterococcus faecalis	-
Serratia marcescens	-
Saccharomyces cerevisiae	-
Candida tropicalis	-
Candida krusei	-
Saccharomyces diastaticus	-
Escherichia coli	-

CHAPTER 4: ISOLATION OF MUTANTS DEFICIENT IN THE SECRETED ANTIBACTERIAL ACTIVITY

4.1 Transposon mutagenesis

In this study transposon mutagenesis (Figure 4.1) was performed to identify gene(s) responsible for the production of the secreted antibacterial activity detected in the CFCS by screening for mutants that no longer secrete this activity, i.e. fail to inhibit the growth of Gram-positive indicator strains.

The initial hypothesis was that if the two activities produced by *S. plymuthica* are related then all or at least some mutants that are selected as being unable to produce the secreted activity should also be deficient in the activity identified on agar plates. On the other hand, if the two activities are different then transposon mutagenesis would result in the production of new mutants; mutants that possess the antibacterial activity detected on agar plates but deficient in the secreted activity.

The transposon was delivered by conjugation between the wild type strain *S. plymuthica* and an *E. coli* strain carrying plasmid pRL27 (4080 bp; Km^R-*ori*R6K) which contains a mini-Tn*5* element (1800 bp) that encodes resistance to kanamycin (Figure 4.2). Preliminary identification of a successful conjugation was done by counting the number of Km^R colonies as illustrated in Figure 4.3.

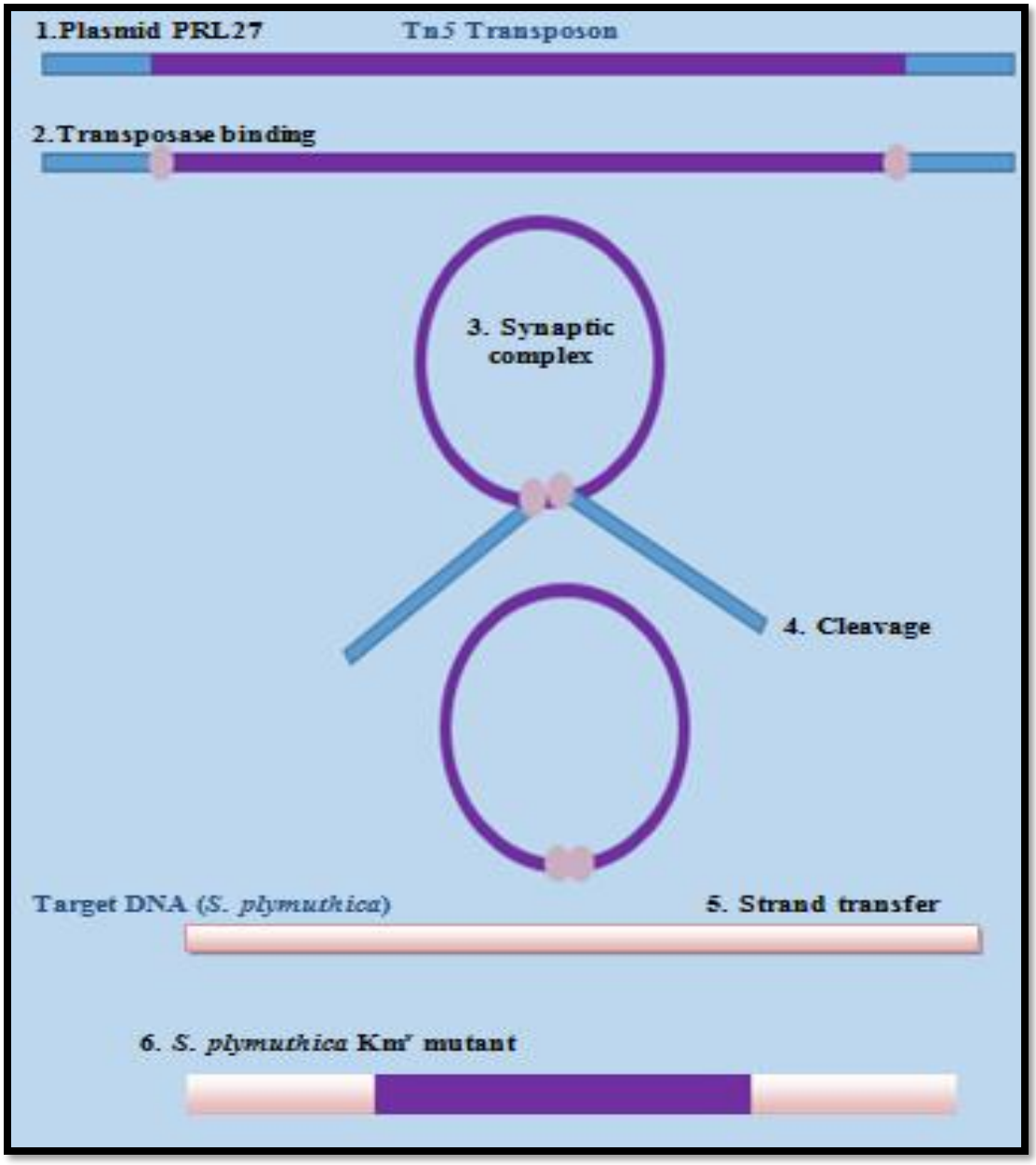

Figure 4.1: An illustration of transposon mutagenesis performed in this study via conjugation between the wild type strain *S. plymuthica* and plasmid pRL27. The plasmid carries a mini-Tn*5* transposon which encodes resistance to kanamycin Km^R. Tn*5* is a composite transposon in which antibiotic resistance genes are flanked by two inverted repeats elements. One of the inverted repeats encodes the protein transposase and both elements are bounded by 19-bp end sequences. Tn*5* transpose via conservative non-replicative cut-and-paste mechanism. Transposition starts when the transposase binds to the 19 bp end sequences leading to an oligomerization reaction and the formation of a synaptic complex. Next, a double strand breakage at both ends of the transposition synaptic complex defined by the end sequences occurs which leads to its release and insertion into the new target. The cleavage of strands at the target site is staggered site creating a characteristic 9 bp target site duplication (TSD) flanking the transposon (Haniford & Ellis, 2015; Reznikoff *et al*., 1999; Bennett, 2008).

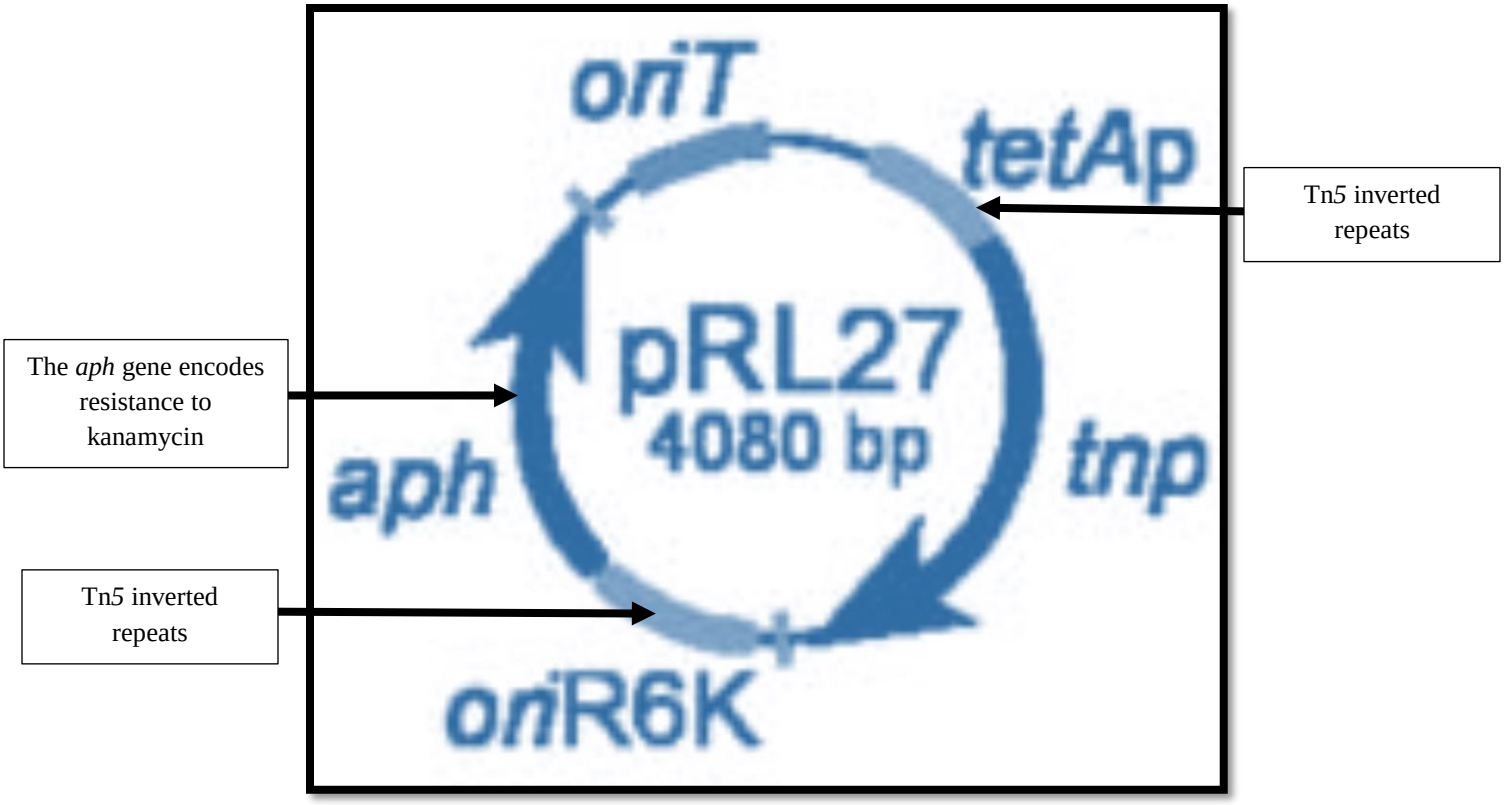

Figure 4.2: Demonstration of plasmid pRL27 used in transposon mutagenesis experiments. pRL27 carries a Tn*5* transposon which encodes resistance to kanamycin and contains an origin of replication (*ori*R6K) that allows cloning of transposon insertion sites. In conjugation the mobilization of the plasmid is via the origin of transfer (*ori*T) (Larsen *et al*., 2002; Hayes, 2003).

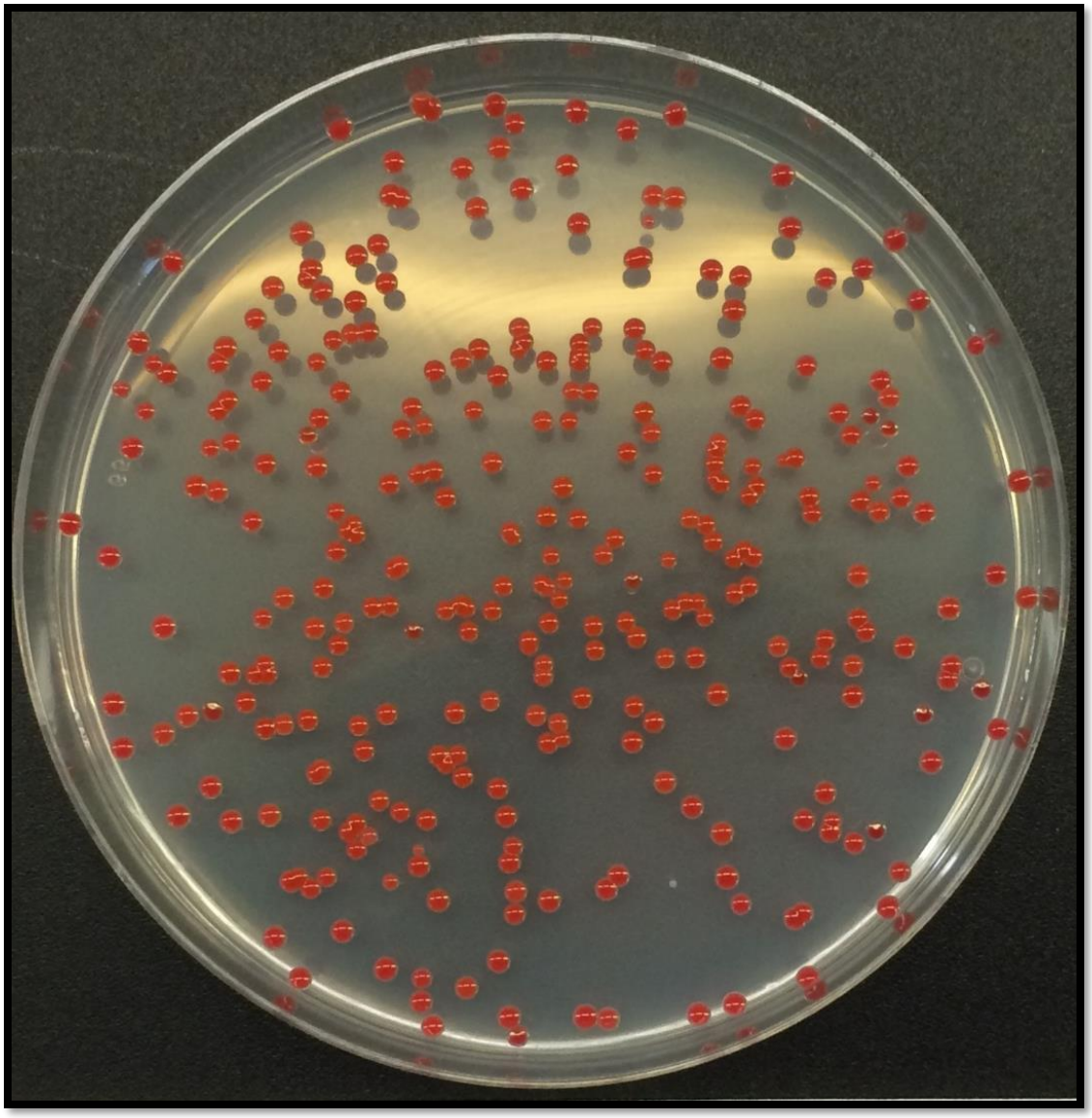

Figure 4.3: A conjugation plate (NA+50µg/ml Kanamycin and 50 µg/ml Ampicillin) of *S. plymuthica* antibiotic resistant transconjugants (24h/RT).

4.2 Screening for mutants deficient in the secreted antibacterial activity

Screening for mutants was preceded by the purification of *S. plymuthica* Km^R colonies. This was done as follows: Km^R mutants were picked aseptically and sub-cultured onto nutrient agar plates+ (50µg/ml Kanamycin) and incubated for 24h at RT. The Km^R colonies were inoculated into centrifuge tubes (50 ml) containing 1 ml of MRS broth without antibiotic; since the transposase (*Tnp*) gene is located outside the inverted repeats thus preventing further transposition, ensuring the stability of the transposon insertion in the target gene (Dennis & Zylstra, 1998). Bacterial cultures of Km^R colonies were incubated at 16^{o}C with shaking 120 rpm for (48h-72h) followed by centrifugation at 3000x g for 15mins. The culture supernatants were collected and sterilized at 70^{o}C for 10mins then left to cool at RT for 15mins. Screening for mutants deficient in secreted antibacterial activity was done by performing spot agar assays against the Gram-positive strains *B. cereus* and (F). The CFCS of *S. plymuthica* Km^R mutants (40 µl) was spotted onto nutrient agar NA square plates pre-spread with 300 µl of a fresh culture of the Gram-positive strains. The wild type *S. plymuthica* was used as positive control (Figure 4.4) and all plates were incubated at RT for 24h (Figure 4.5). Mutants deficient in the secreted antibacterial activity; lack of zones of inhibition against the indicator strains were isolated and subjected to further tests (Figure 4.6).

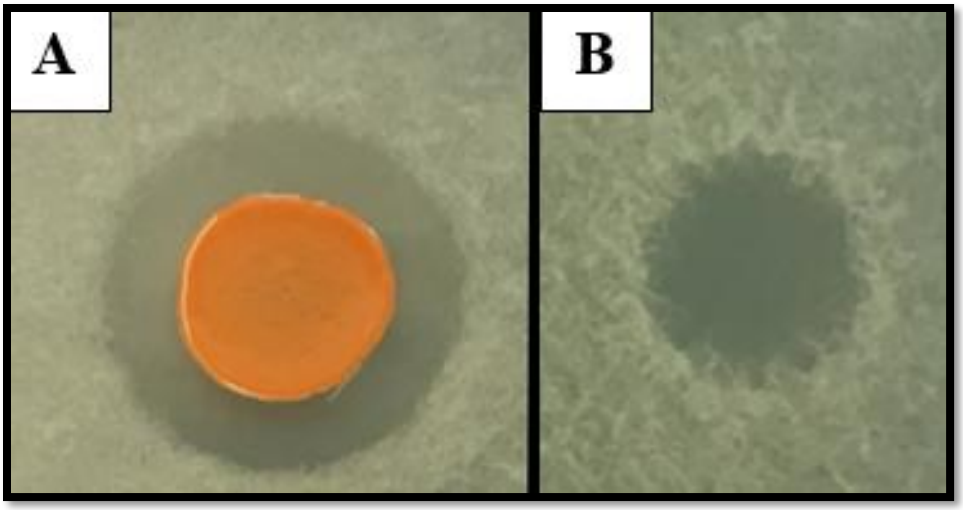

Figure 4.4: The phenotypic bioactivity of the environmental strain *S. plymuthica* against the indicator strain (F) NA; (24h/ RT). A; Production of inhibitory activity when *S. plymuthica* is grown on plates. B; Presence of inhibitory activity in the CFCS.

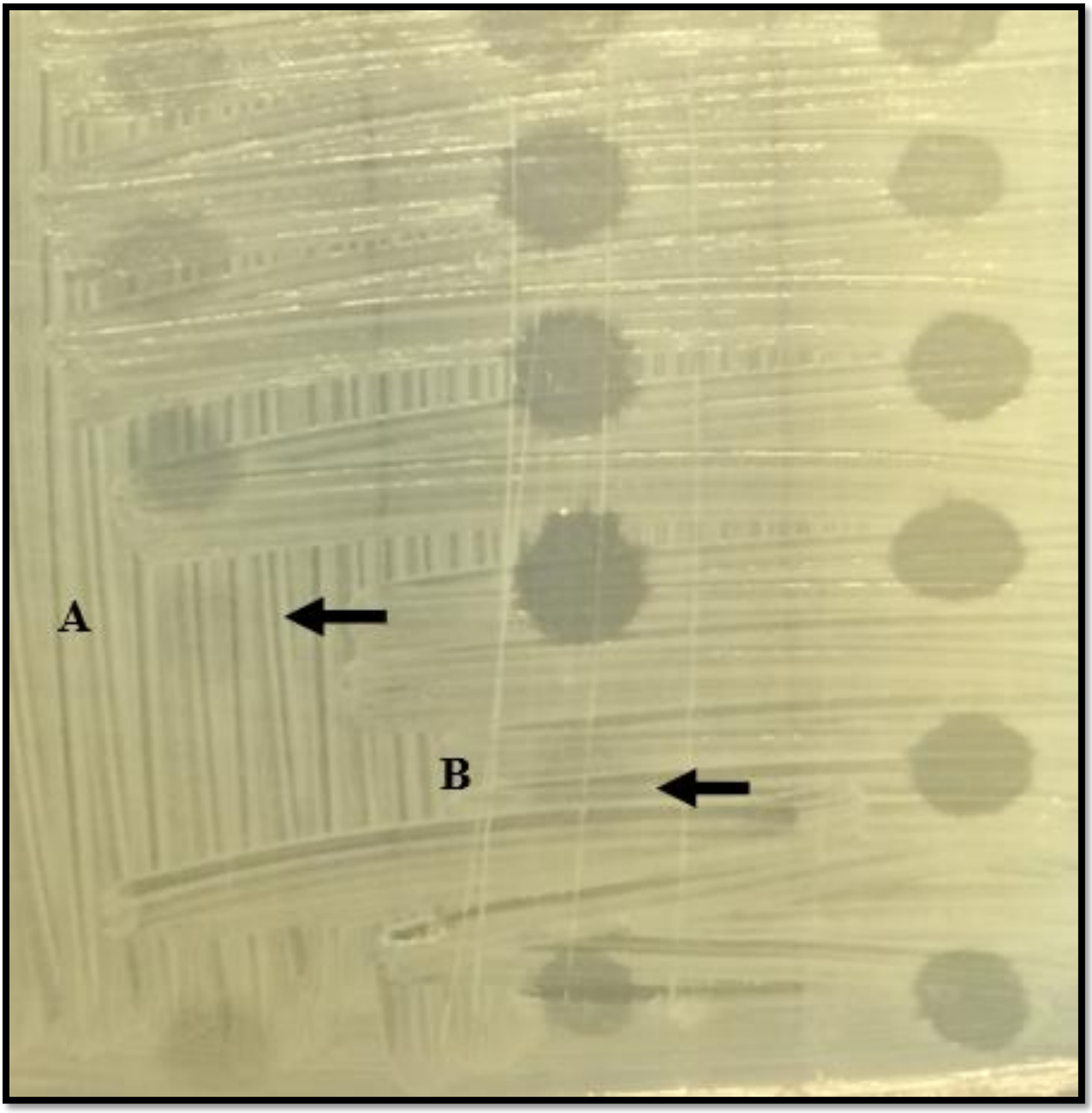

Figure 4.5: The results of screening for mutants deficient in the secreted antibacterial activity. *S. plymuthica* Km^R mutants were inoculated in MRS broth; (16°C/48-72h). Mutants with a CFCS deficient in antibacterial activity; lack of zone of inhibition against the indicator strain *B. cereus*; NA(24h/RT) were considered potential mutants and subjected to further tests. labeled as A and B and by the arrows.

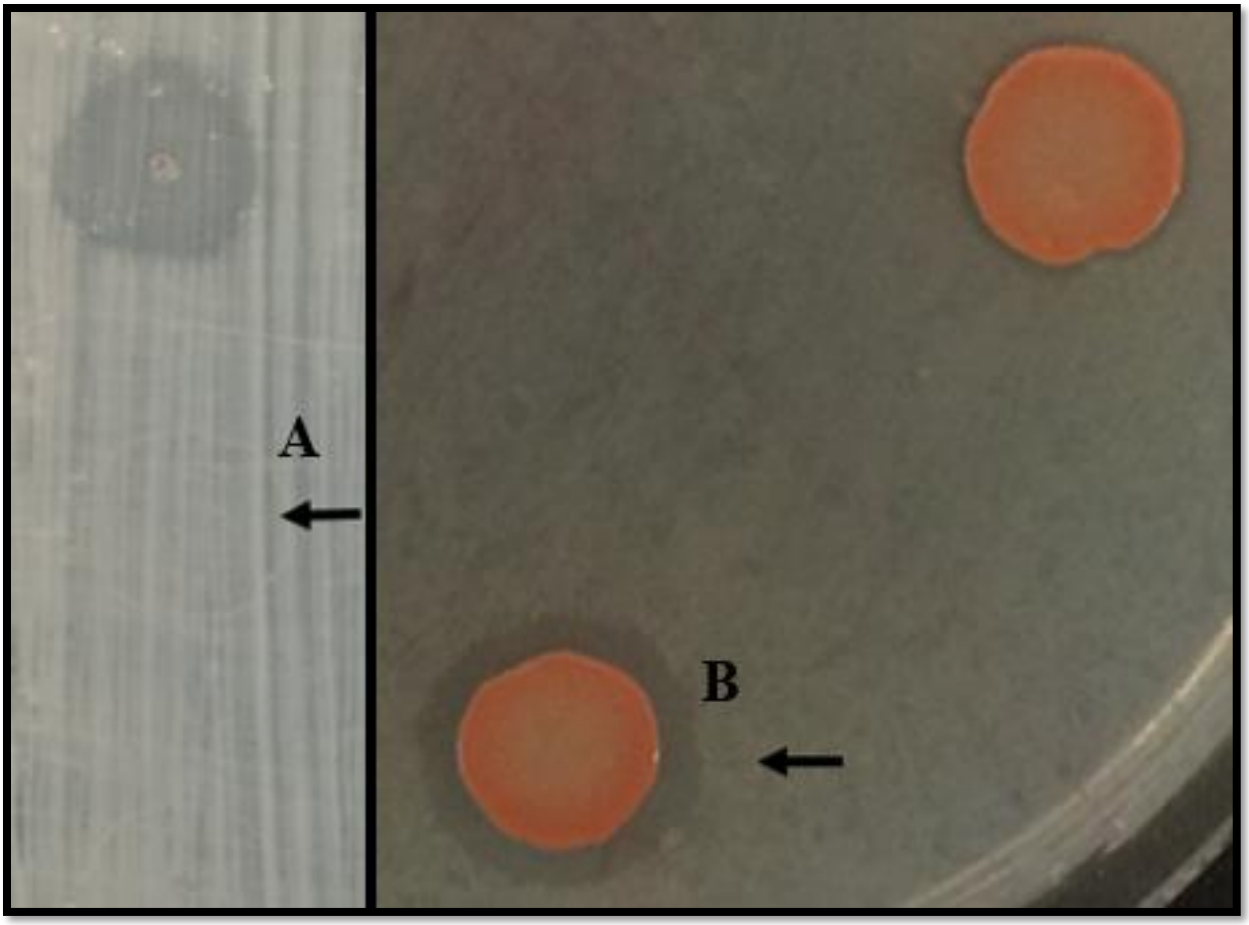

Figure 4.6: *S. plymuthica* Km^R mutants deficient in the secreted antibacterial activity (A) were subjected to further tests. Those mutants were inoculated in NB broth and incubated at (RT/18h) with shaking 120 rpm. Mutants that produce antibacterial activity (B) against the indicator strain *B. cereus*; NA(24h/RT) were considered potential mutants.

In this study, 597 Km^R transposon induced mutants were screened resulting in the isolation of two non-antibacterial producing strains named; M74 and M426. Those mutants lacked the secreted antibacterial activity in their CFCS. The Mutant strains exhibited different colony morphology, mutant M74 was pigmented as the parent strain and produced pink glistening colonies (Figure 4.7), while mutant M426 was non- pigmented and produced white mucoid colonies (Figures 4.8).

In order to test the hypothesis previously mentioned in Section 4.1; the ability of the Km^R transposon induced mutants M74 and M426 to produce antibacterial activity when grown on agar plates. This was done as follows: mutants M74 and M426 were inoculated in NB broth and incubated at RT for 18h with shaking 120 rpm and spot agar assays were performed using the mutants bacterial culture against the Gram-positive strains. Plates were incubated at RT for 24h.

Results showed the presence of inhibitory activity against the indicator strain. The phenotypic activities of M74 and M426 are demonstrated in Figures 4.9 and 4.10.

For further confirmation, mutants deficient in antibacterial activity detected on agar plates obtained by AlThubiani, (2013), were tested against the Gram-positive strains using the mutants' CFCS. Those mutants showed activity against the Gram-positive strains (Data not shown). These results indicate that *S. plymuthica* produce multiple antimicrobial compounds and the two observed activities are distinct.

Figure 4.7: Colony morphology of Km^R transposon induced mutant M74 compromising small pink glistening colonies on nutrient agar plate NA after growing for 24hr at room temperature RT.

Figure 4.8: Colony morphology of Km^R transposon induced mutant M426 compromising small white mucoid colonies on nutrient agar plate NA after growing for 24h at room temperature RT.

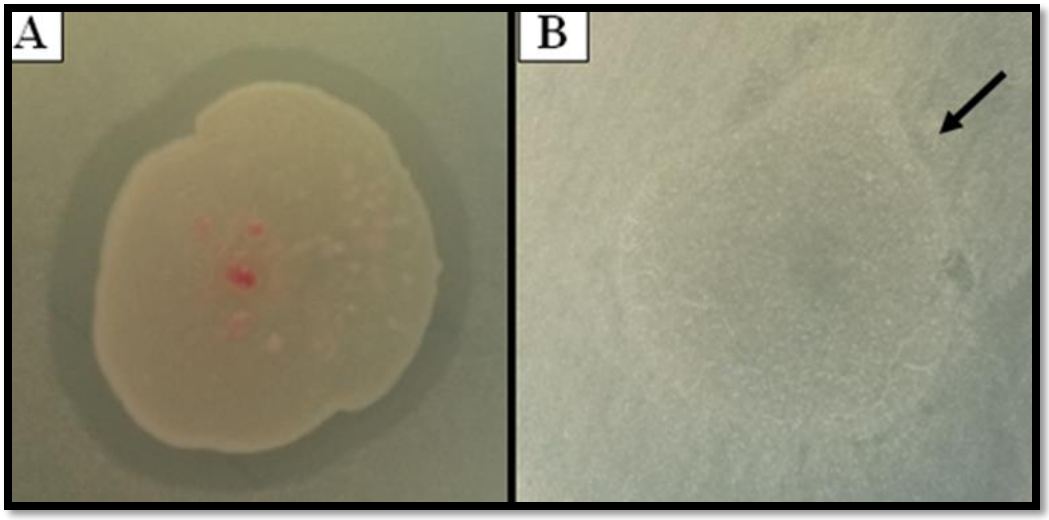

Figure 4.9: The phenotypic bioactivity of the Km^R transposon induced mutant M74 obtained in this study against the indicator strain *B. cereus*; NA; (24h/ RT). A; inhibitory activity produced by the mutant when grown on plates. B; lack of inhibitory activity in the mutant's CFCS.

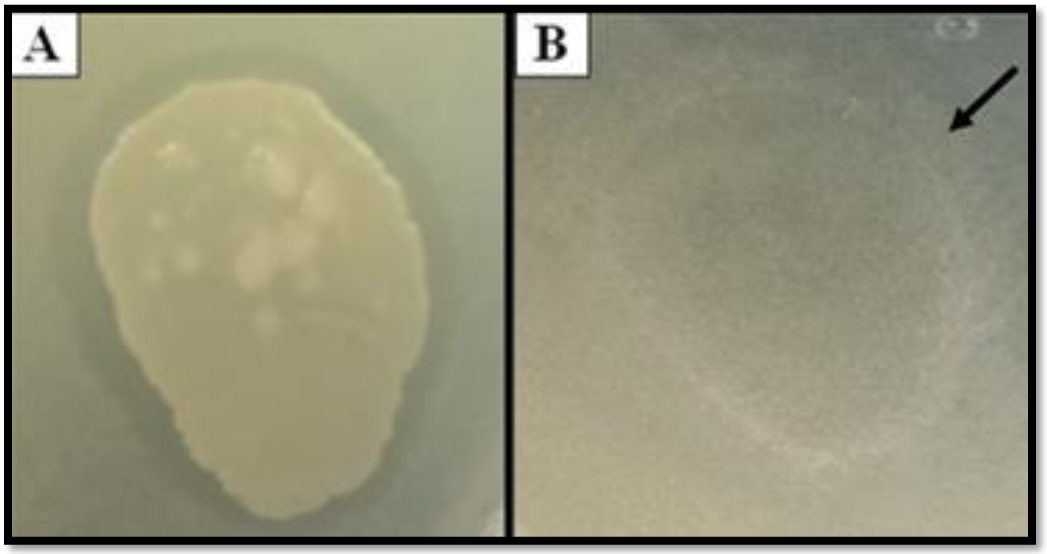

Figure 4.10: The phenotypic bioactivity of Km^R transposon induced mutant M426 against the Gram-positive strain *B. cereus*; NA; (24h/ RT). A; inhibitory activity produced by the mutant when grown on plates. B; lack of activity in the CFCS.

4.3 Detection of the Km^R gene

To verify that the observed phenotype of each mutant; M74 and M426 was indeed caused by Tn*5* transposon insertion; insertion of the Km^R gene into the genome of *S. plymuthica*. A PCR reaction was performed as previously described in Section 2.15 using primers (AGPT-F and AGPT-R) that anneal to the Km^R gene (Larsen *et al*., 2002).

The genomic DNA of the mutant strains was used as a template. Controls were also included; a positive control (plasmid PRL27, containing the Km^R gene) and a negative control (genomic DNA of the wild type). Amplified PCR products were visualized via agarose gel electrophoresis as mentioned in Section 2.14.3 and a 1kb DNA Ladder was used as a marker.

Results of gel electrophoresis of the PCR products showed the presence of bands measuring ~600 bp corresponding to the PCR products of mutants M74, M426 and plasmid pRL27 (positive control) and confirming the presence of the Km^R gene within the genomic DNA of the mutants. As expected, the PCR product of the wild type strain did not produce a band on gel electrophoresis as shown in the following figure:

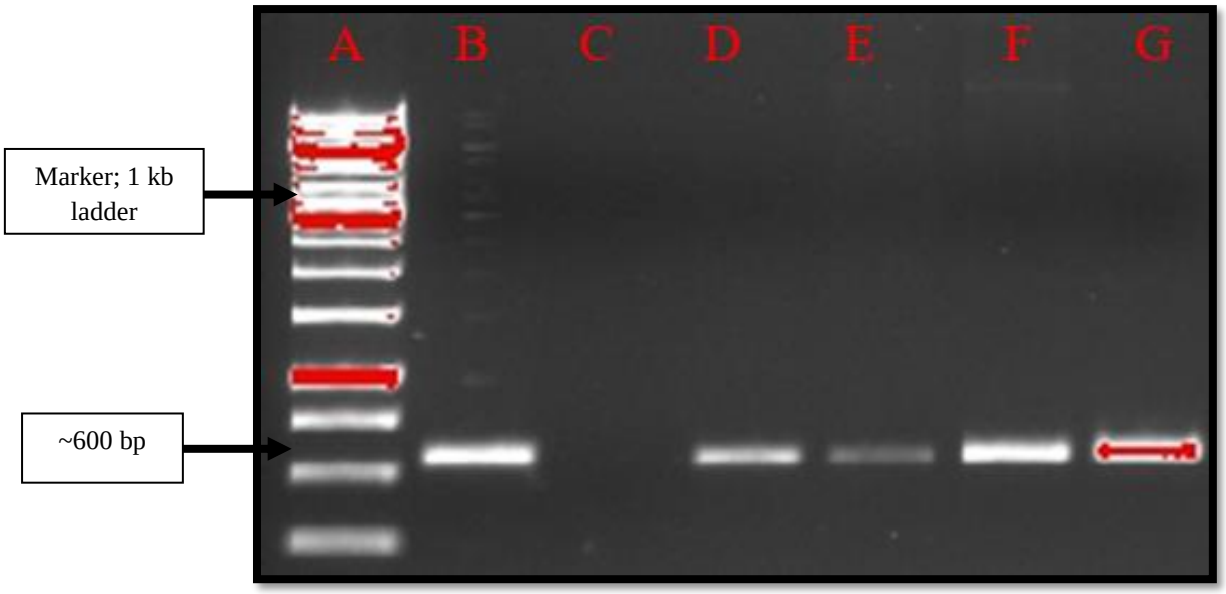

Figure 4.11: Agarose gel electrophoresis of the amplified Km^R gene. A; 1 kb ladder, B; PCR product of pRL27 (positive control), C; PCR product of the wild type strain *S. plymuthica* (negative control), lanes D and E; PCR products of Km^R mutant M74, lanes F and G; PCR products of Km^R mutant M426.

4.4 Cloning and identification of transposon insertion sites

Cloning and recovery of the transposon insertion and the flanking DNA was performed in order to identify gene(s) responsible for the production of the secreted antibacterial activity detected in the CFCS. This was done as follows: the genomic DNA of Km^R mutants M74 and M426 was isolated using the Thermo Scientific GeneJET Genomic DNA Purification Kit as previously described in Section 2.14.1 and was followed by restriction enzyme digestion using the enzyme (*Bam*HI) which does not cut within the transposon in order to produce a DNA fragment with the oriR6K origin, the *aph* gene and adjacent genomic DNA sequences. The digested DNA was subjected to a ligation reaction using the enzyme T4 DNA ligase which promotes self-ligation of the *Bam*HI DNA fragments (Larsen *et al*., 2002). The efficiency of the methods was confirmed by the visualization of DNA via agarose gel electrophoresis. Both Km^R mutants produced bands on agarose gels after Genomic DNA extraction and a smear after enzymatic digestion with *Bam*HI as shown in the following figure:

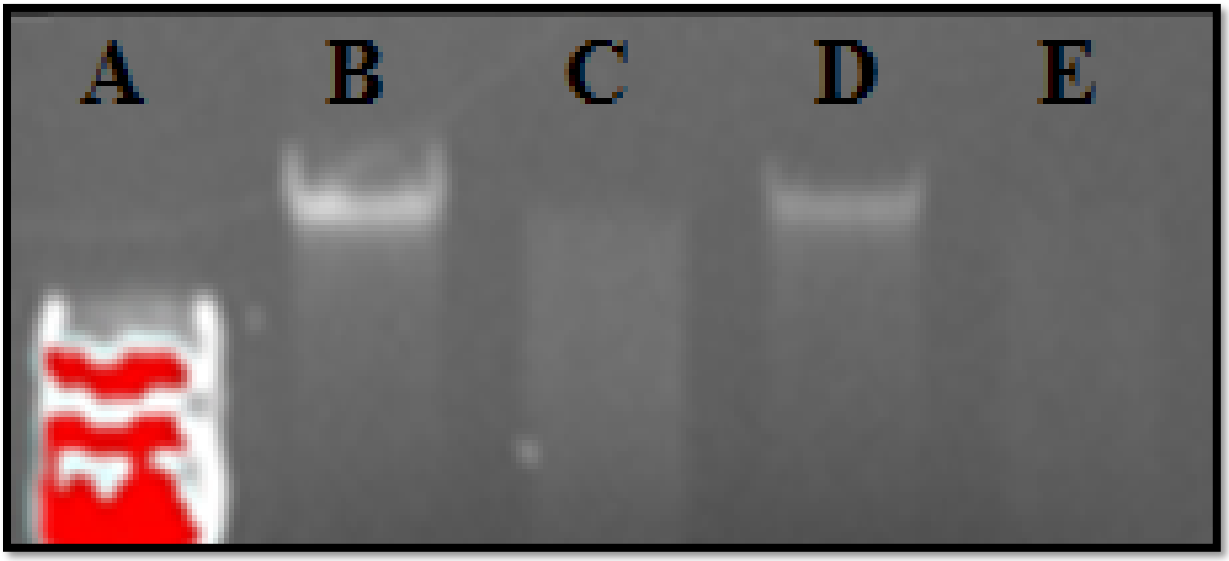

Figure 4.12: Agarose gel electrophoresis of km^R mutants obtained in this study after Genomic DNA extraction and enzymatic digestion with *Bam*HI. A; 1 Kb ladder, B; Genomic DNA of mutant M74, C; digested Genomic DNA of mutant M74), D; Genomic DNA of mutant M426, E; digested Genomic DNA of mutant (M426).

After performing ligation reactions on digested genomic DNA of Km^R mutants M74 and M426, Ligated DNA was introduced into the *E. coli* strain S17-1 λpir via electroporation. This strain contains the *pir* gene which allows circularized DNA fragments containing the transposon to replicate as *pir*-dependent plasmids (Kvitko *et al*., 2012).

Despite several repeated attempts to clone M74 and M426 using home-made competent cells, cloning experiments were not successful. Hence, commercially available cells (TransforMax™ EC100D™ *pir*-116 electrocompetent *E. coli*) were used instead. However, cloning experiments were still not successful. Following this, the restriction enzyme *Eco*RI was used instead of *Bam*HI in an attempt to identify and clone transposon insertion sites. It should be noted that there is an *Eco*RI site inside the right-side end of the Tn*5* which does not cut inside the transposon flanking sequences allowing directional cloning (Tolonen *et al*., 2006; Choi *et al*., 2012; Erb *et al*., 2010; Salch & and Shaw, 1988).

The genomic DNA of Km^R mutants M74 and M426 were extracted as previously mentioned and enzymatically digested with *Eco*RI. The efficiency of the methods was confirmed via agarose gel electrophoresis (Figure 4.14). The Ligated- cut Genomic DNA of M74 and M426 was introduced into TransforMax™ EC100D™ *pir*-116 electrocompetent *E. coli* via electroporation as described in Section 2.19.3 and the following control provided by the manufacturer was included; [Transformation control (116+)].

Using sterile glass beads, all samples; (100µl each) were plated onto LB plates supplemented with the antibiotic Km; (50µg/ml) and incubated for 24h at 37^{o}C. The produced colonies were purified by sub-culturing onto new LB plates+ Km. Recombinant plasmids form Km^R *E. coli* transformants cells were inoculated in LB broth+ km; 50µg/ml and incubated for 24h at 37^{o}C followed by purification of plasmid DNA using the Thermo Scientific GeneJET Plasmid Miniprep Kit. Enzymatic digestion using *Eco*R1 was performed on the recombinant plasmids of mutants M74 and M426 in order to confirm the presence of the genomic DNA flanking the transposon insertion site in *pir*-116 *E. coli*. Agarose gel electrophoresis of the digested recombinant plasmids confirmed a successful delivery of Tn5 into *pir*-116 *E. coli* (Figure 4.15). Identification of transposon insertion sites was preceded by DNA sequencing at Edinburgh Genomics. This was done as described in Section 2.20.2 using primers TPNRL17-1 and TPNRL13-2 that anneal to the origin of replication and the Km^R gene of the transposon (Figure 4.16) (Bruckbauer *et al*., 2015; Zhang *et al*., 2000; Buchan *et al*., 2008).

Only DNA sequencing of the recombinant plasmid from M426 was successful. Nucleotide sequences of DNA segment flanking the transposon insertion was compared with those in the databases of the National Center for Biotechnology Information (NCBI) using the query BLASTX. Results showed that the Tn5 has inserted into a gene that encodes a PKSs with 70% identity (Table 4.1). Amino acid sequence of Polyketide synthase of the flanking region of transposon insertion of mutant M426 reveals how well the query sequence matches the subject sequence in the database (Figure 4.17).

Based on the above results a conclusion was drawn with two possibilities regarding the antibacterial activity of the wild type strain: (1) The presence of two separate biochemical pathways leading to two distinct activities; the activity detected on agar plates and the other secreted activity detected in the CFCS (2) There is a branched biochemical pathway with one branch leading to the activity observed on the plates and another branch leading to the secreted activity. All steps leading to cloning and identification of transposon insertion site are illustrated in Figure 4.13.

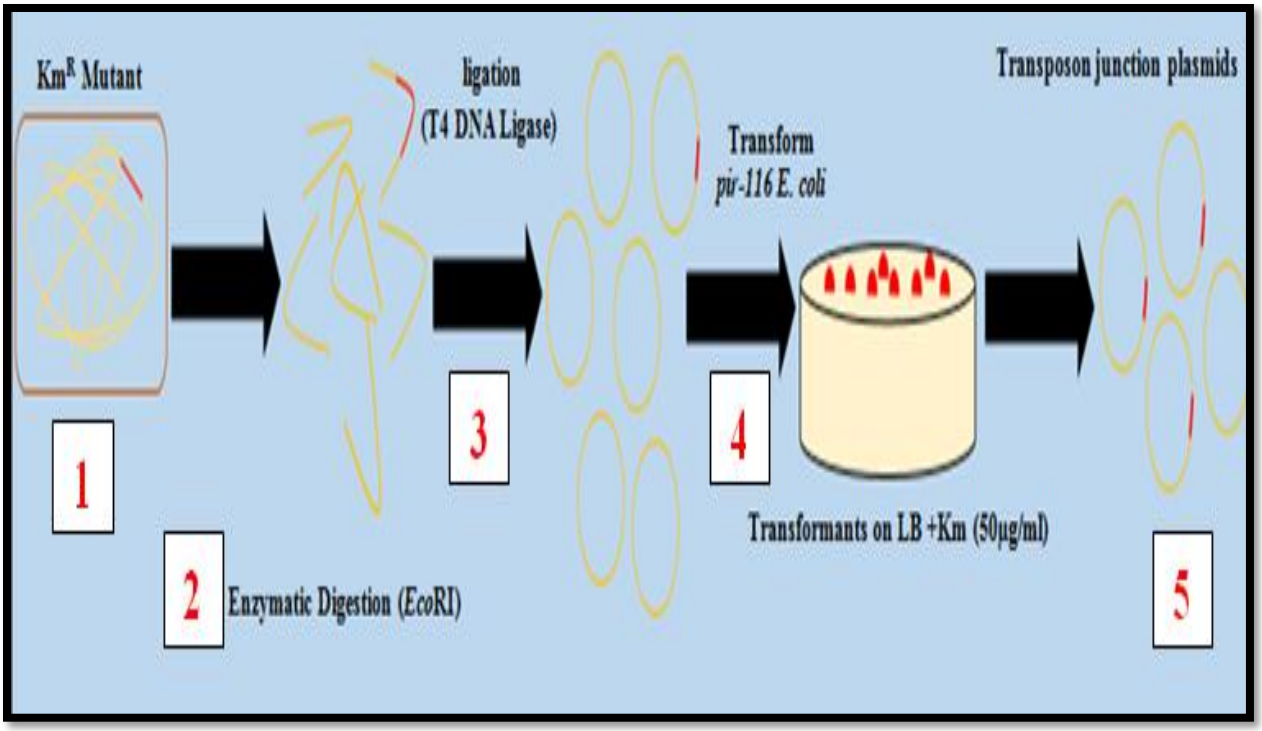

Figure 4.13: An illustration of the steps of cloning and identification of transposon insertion sites. The genomic DNA of Km^R mutant M426 was extracted followed by enzymatic digestion with *Eco*RI. Ligated Genomic DNA was introduced into TransforMax™ EC100D™ *pir*-116 electrocompetent *E. coli* via electroporation. The samples were plated onto LB plates +Km; (50µg/ml) followed by incubation (24h/37ºC). Recombinant plasmids form Km^R *E. coli* transformants cells were purified in order to be sequenced using primers TPNRL17-1 and TPNRL13-2 that anneal to the origin of replication and the Km^R gene of the transposon.

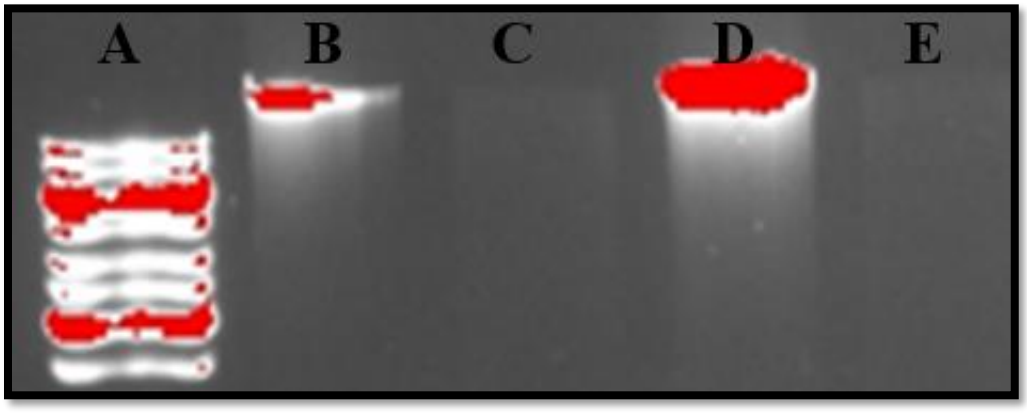

Figure 4.14: Agarose gel electrophoresis of Km^R mutants obtained in this study after Genomic DNA extraction and *Eco*RI digestion. A; 1 Kb ladder, B; Genomic DNA of mutant M74, C; digested Genomic DNA of mutant M74, D; Genomic DNA of mutant M 426, E; digested Genomic DNA of mutant M426.

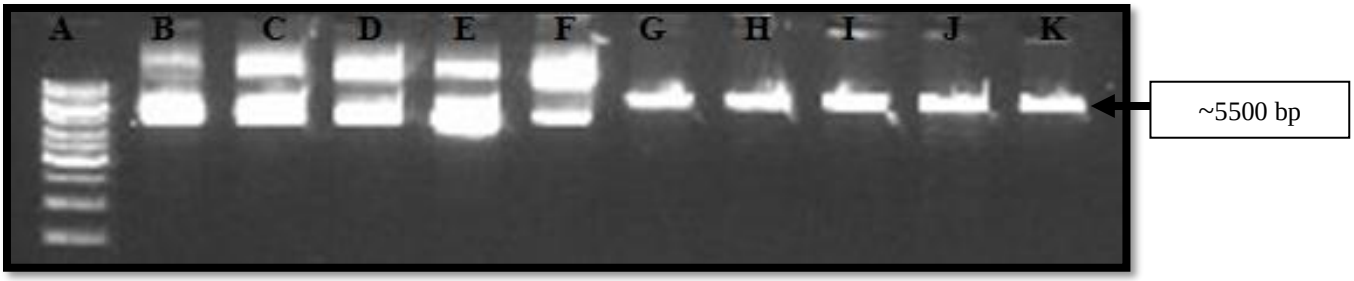

Figure 4.15: Agarose gel electrophoresis of *Eco*RI uncut and cut recombinant plasmids (WP1-WP5) of mutant M426. Enzymatic digestion was performed on recombinant plasmids in order to confirm the presence of the transposon insert in the vector *pir*-116 *E. coli*. Supercoiled DNA run faster than linear DNA making it difficult to determine the size of supercoiled DNA. Hence, a lower limit on the size is given. A; 1 Kb ladder. Lanes (B-F); uncut transposon recombinant plasmids (WP1-WP5), Lanes (G-K); cut transposon recombinant plasmids WP1-WP5.

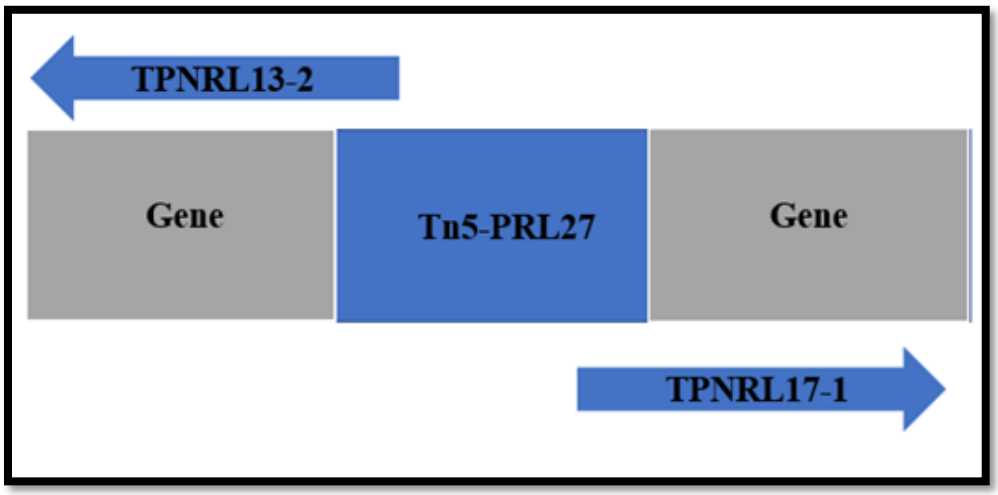

Figure 4.16: Partial sequencing of the flanking genomic DNA of Km^R recombinant plasmids obtained in this study was done using primers TPNRL17-1 and TPNRL13-2 that anneal to the origin of replication and the Km^R gene of the transposon.

Table 4.1: Results of BLASTX search obtained from the partial sequencing of the flanking genomic DNA of mutant M426 showing that the Tn*5* has inserted into a gene resembling PKS

Description	Max score	Total score	Query cover	E value	Max identity	Accession
Polyketide synthase (*Paenibacillus peoriae*)	91.7	91.7	62%	9e-20	70%	WP_076265426.1
Polyketide synthase (*Paenibacillus peoriae*)	91.7	91.7	62%	9e-20	70%	WP_075154433.1
Polyketide synthase (*Paenibacillus peoriae*)	91.7	91.7	62%	9e-20	70%	WP_064962241.1

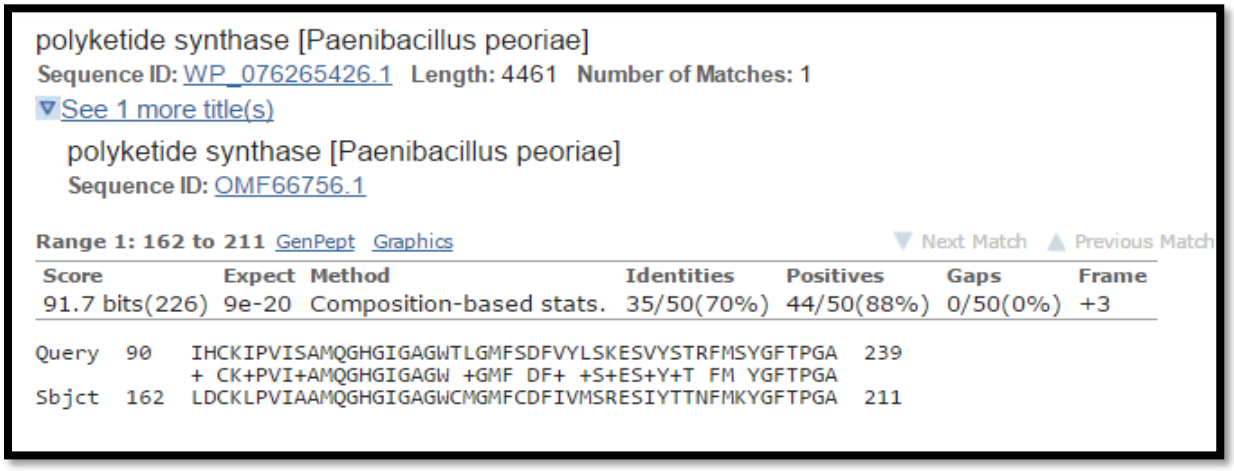

Figure 4.17: Amino acid sequence of Polyketide synthase obtained from Blastx analysis (NCBI) of the flanking region of transposon insertion of mutant M426 using TPNRL17-1 primer in a comparison with the database.

CHAPTER 5: CHEMICAL PROFILING OF THE SECRETED ANTIBACTERIAL ACTIVITY

5.1 Concentration of secreted antibacterial activity

In order to further investigate the secreted antibacterial activity, freeze-drying was performed to concentrate the CFCS. This was done as follows: a fresh sample of the CFCS (50 ml) was distributed in shallow plates and placed at -20^{o}C for 24h in preparation for freeze drying as previously mentioned in Section 2.21.2. A control was included; (50 ml of NB only). Concentrating the CFCS resulted in the production of a powder-like material (Figure 5.1) which was then re-suspended in 2 ml sterile deionized water; (resulting in a 25-fold concentration) and named concentrated culture supernatant or CCS. To avoid the presence of contamination, the re-suspended CCS was sterilized using a 0.2 µm membrane filter and the presence of activity was assessed against the Gram-positive strain (F). The CCS produced activity with an inhibition zone diameter of 11.3 mm while, the negative control (concentrated NB) did not produce any activity (Figure 5.2).

In this study, one of the initial attempts to concentrate the culture supernatant was by performing rotary evaporation as previously mentioned in Section 2. 21.1. The end result of this method was a brown gel-like material (Figure 5.3) which was removed and re-suspended in 2 ml of sterilized deionized water and stored at 4^{o}C until needed. The presence of activity was assessed as previously mentioned above. The rotary evaporation concentrate produced activity with an inhibition zone diameter of 21.0 mm. To make sure that the white growth in the middle of the zone of inhibition is a precipitant and not contamination using a sterile cotton swab, the rotary evaporation concentrate was streaked onto an NA plate and incubated for 24h at RT. There was no presence of growth or colonies (Data not shown). Unfortunately, there was some problems regarding this method, it was time consuming; (5h) and it was difficult to remove the brown gel-like material from the rotary bulb due to its high viscosity. For these reasons, freeze drying was used as the main method to concentrate the CFCS.

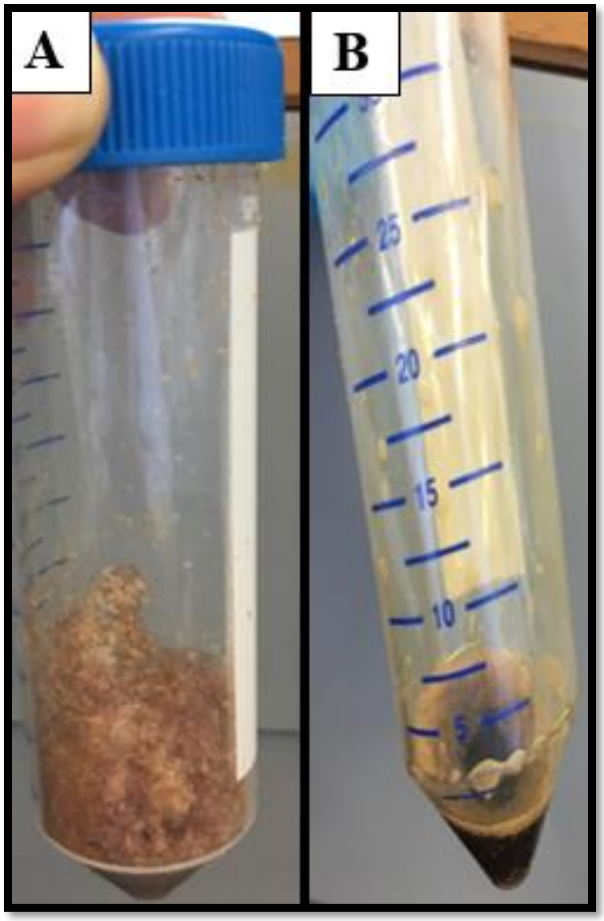

Figure 5.1: Demonstration of the end-product of concentrating the CFCS. Concentrating the CFCS via freeze-drying resulted in the production of a powder-like material (A) which was re-suspended in 2 ml sterile deionized water and named concentrated culture supernatant CCS (B).

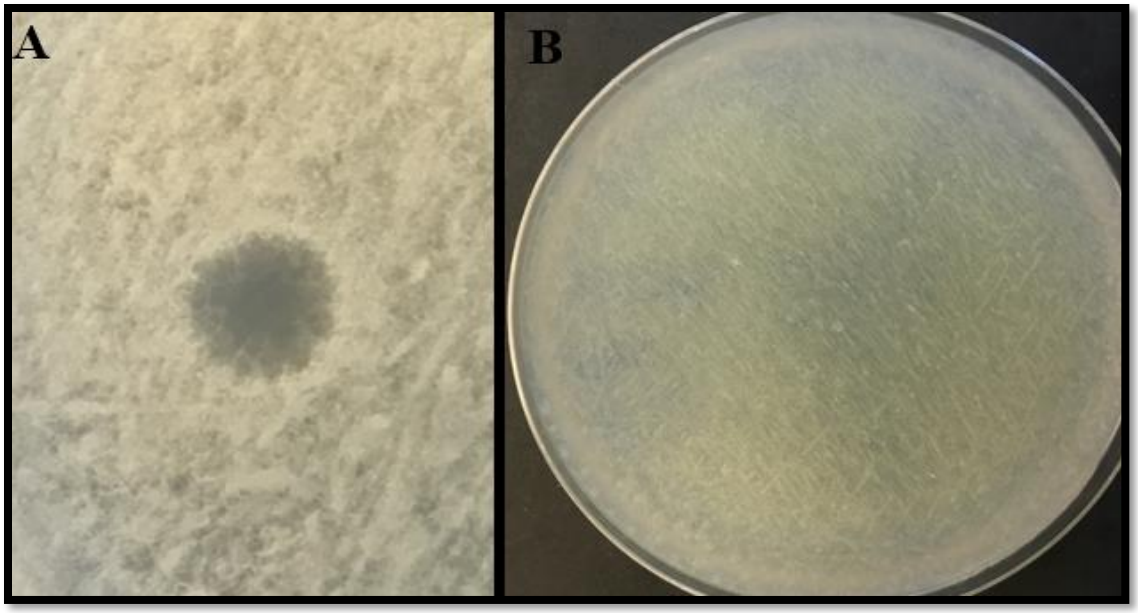

Figure 5.2: Demonstration of the antibacterial activity of the CCS against the Gram-positive strain (F); NA (RT/24h). A; zone of inhibition (12.0 mm) in diameter produced by the CCS. B; a nutrient agar plate showing lack of activity produced by the concentrated negative control (NB broth only) against the Gram-positive strain. This image is a representative image of 3 replicates.

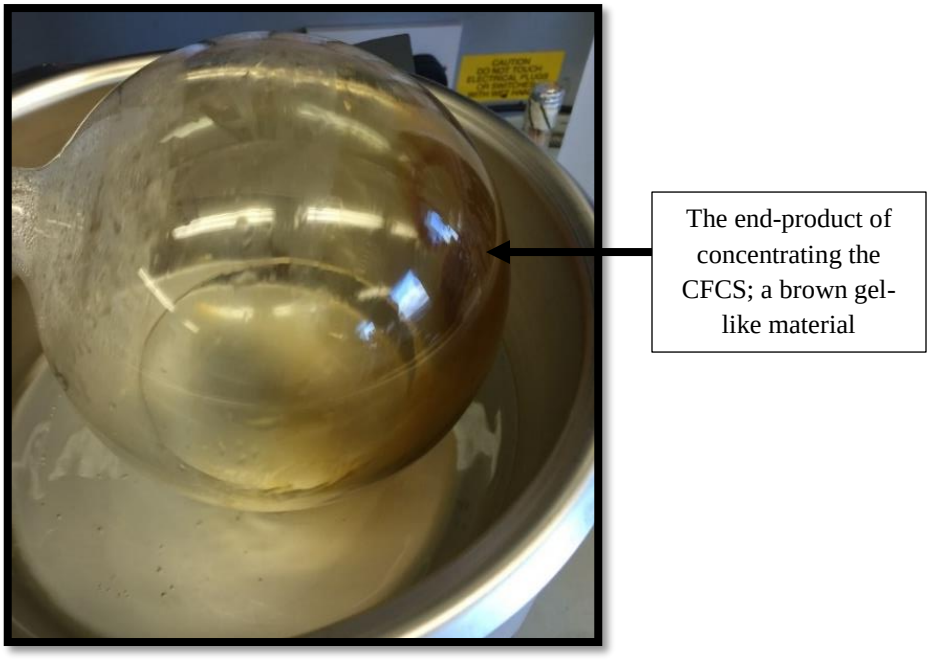

Figure 5.3: Demonstration of the result of concentrating the CFCS via rotary evaporation and the production of a brown gel-like material which was collected from the rotary bulb and re-suspended in sterile deionized water (2 ml).

5.2 Purification of the secreted antibacterial compound(s) by organic solvent extraction

The secreted antibacterial compound(s) present in the CFCS was purified using methanol as previously mentioned in Section 2.22. Centrifugation resulted in the production of supernatants and pellets in all samples which were later named soluble and insoluble fractions respectively. The soluble fractions were placed into new centrifuge tubes before uploading all the samples into the ThermoSavant AES2010 SpeedVac® System to be evaporated to dryness in order to eliminate any traces of methanol. Sterile deionized water (1 ml) was added to all samples and the presence of activity was assessed against the Gram-positive strain (F).

Results showed that all samples, excluding that of the negative control; (methanol directly spotted onto the plate) produced activity against the gram-positive strain; indicating the efficiency of the evaporation step (Table 5.1). The soluble and insoluble fractions of the positive control produced activity against the Gram-positive strain with inhibition zone diameters of 10.0 and 10.6 mm respectively (Figure 5.4). While, the soluble and insoluble fractions of the CCS produced activity with inhibition zone diameters of 12.3 and 11.6 mm respectively (Figure 5.5 and 5.6). These results might indicate that the secreted antibacterial compound(s) can aggregate or the presence of two secreted antibacterial compounds in the CFCS, one that is insoluble in methanol while the other is soluble in methanol.

Table 5.1: The inhibitory activity of the purified secreted antibacterial compound(s). The inhibitory activity was assessed against the Gram-positive strain (F). Inhibition zones are measured in millimeters. Data shown are the means ± SD, n=3

Fractions	Inhibition zones
Positive control (soluble fraction)	10.0 ± 0
Positive control (insoluble fraction)	10.6 ± 1.1
CCS (soluble fraction)	12.3 ± 1.1
CCS (insoluble fraction)	11.6 ± 0.5
Negative control (methanol only)	0

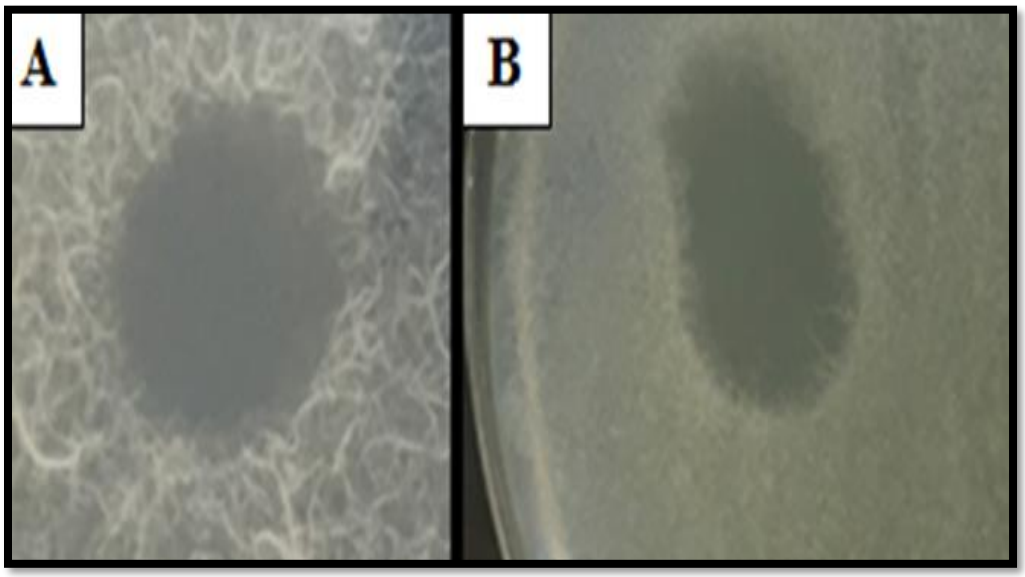

Figure 5.4: The activity of the positive controls used in organic solvent extraction method against the Gram-positive strain (F); NA (RT/24h). A; a zone of inhibition (12.0 mm) in diameter produced by the insoluble fraction of the positive control. B; a zone of inhibition (10.0 mm) in diameter produced by soluble fraction of the positive control. This image is a representative image of 3 replicates.

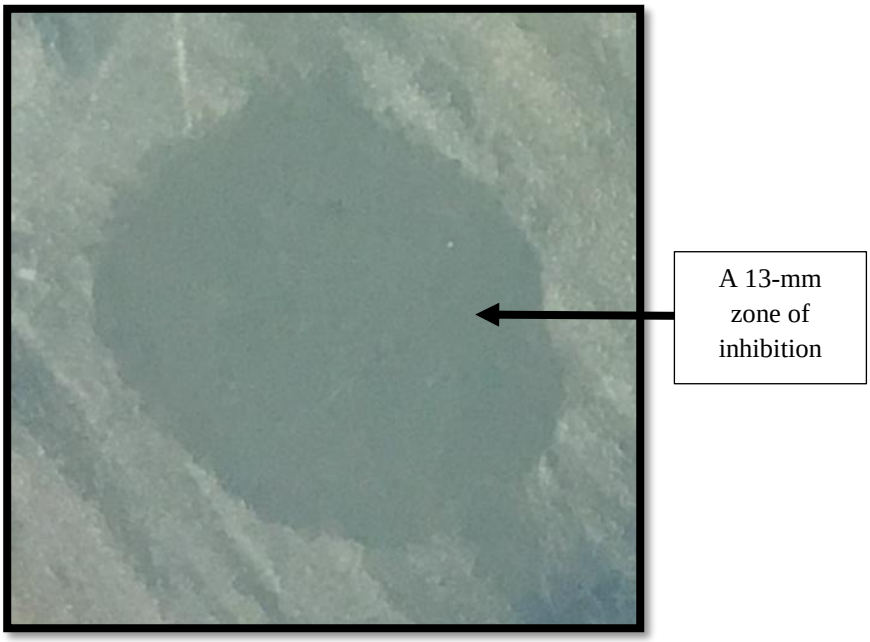

Figure 5.5: The activity of the soluble fraction of the CCS against the Gram-positive strain (F). This image is a representative image of 3 replicates.

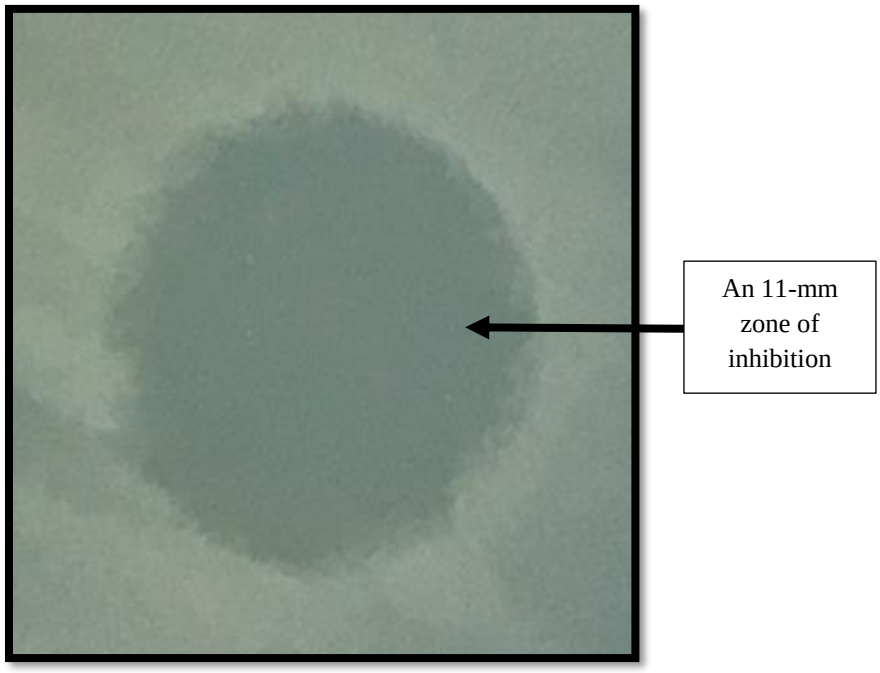

Figure 5.6: The activity of the insoluble fraction of the CCS against the Gram-positive strain (F). This image is a representative image of 3 replicates.

5.3 NMR spectroscopy

To get a closer understanding of the secreted antibacterial compound(s) an NMR was performed as follows: the activity of the CFCS and the CCS was initially assessed against the Gram-positive strain *Bacillus cereus*. Both samples produced activity with inhibition zones diameter of 10.0 mm. The CFCS and the CCS were mixed with equal volumes of methanol followed by centrifugation at (3000xg/15mins). The supernatants were placed into new centrifuge tubes and all samples were loaded into the ThermoSavant AES2010 SpeedVac® System to be evaporated to dryness in order to eliminate any traces of methanol. For the purpose of this experiment, the samples were named as follows: sample 1(soluble fraction of the CFCS), sample 2 (insoluble fraction of the CFCS), sample 3 (soluble fraction of the CCS) and sample 4 (insoluble fraction of the CCS). The samples were re-dissolved in 600 µl of DMSO in order to avoid the spectra to be dominated by the solvent signal then transferred to NMR tubes (5 mm) before analysis using JEOL AS400 NMR spectrometer. The spectrum of all samples showed a peak at 2.5 ppm corresponding to the deuterated solvent DMSO-d_6 (Figure 5.7).

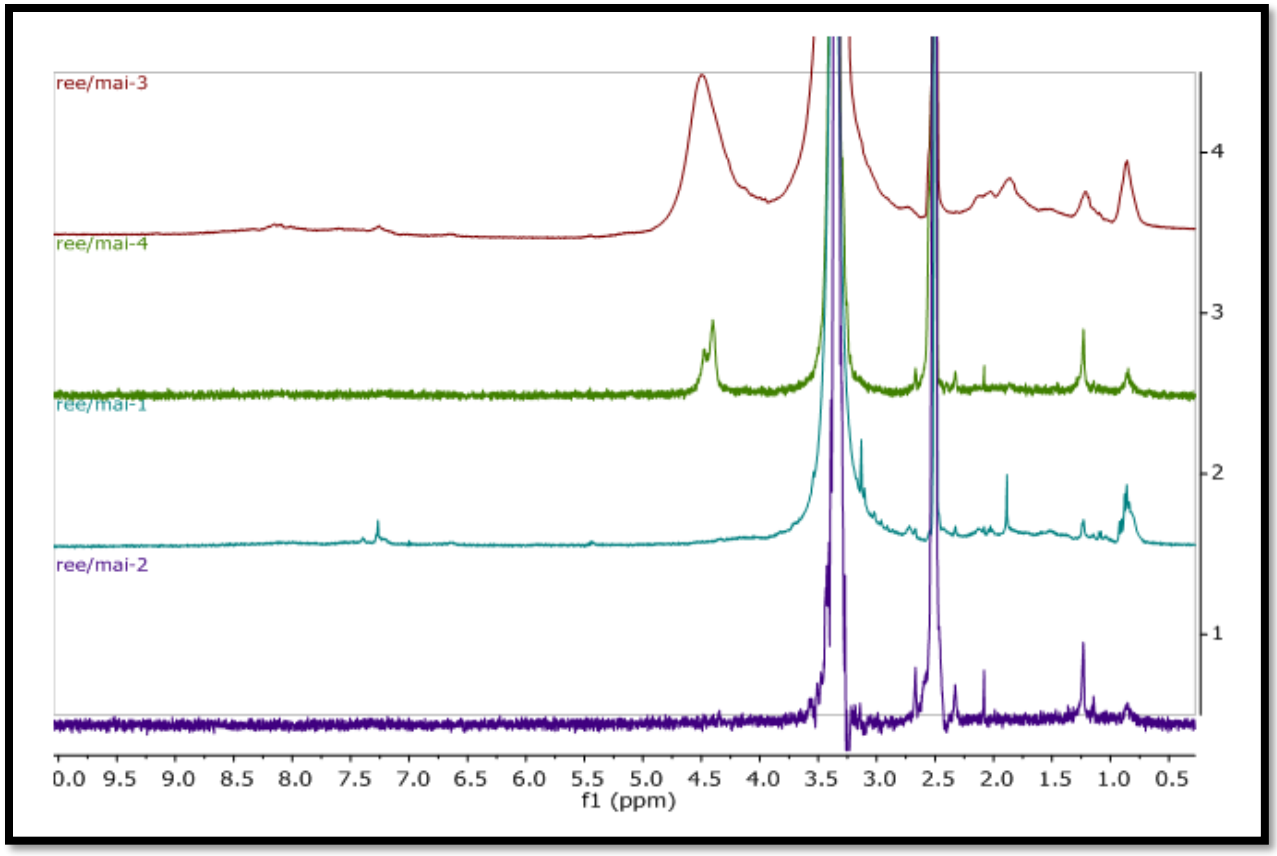

Figure 5.7: The NMR spectrum of the secreted antimicrobial compound(s) have two dimensions: the x axis corresponding to the frequency (ppm) and the Y axis corresponding to the intensity. The peak at 2.5 ppm is the DMSO signal. Samples were named as follows: [sample 1; soluble fraction of the CFCS, sample 2; insoluble fraction of the CFCS, sample 3; soluble fraction of the CCS and sample 4; insoluble fraction of the CCS]. The NMR spectrum of sample 3 showed peaks with high intensity at 3-5 ppm and peaks with smaller intensity at 7-8 ppm and 0-1 ppm corresponding to a polyketide structure. The NMR spectrum of sample1 showed peaks at 1-1.5 ppm and 3-3.5 ppm corresponding to a polyketide structure. The NMR spectrum of samples 2 and 4 showed peaks at 2.5 ppm corresponding to the DMSO. All other peaks shown on the spectra above did not correspond to any known compounds of interest.

The NMR spectrum of sample 3 showed peaks with high intensity at 3.0-5.0 ppm and peaks with smaller intensity at 7.0-8.0 ppm and 0-1.0 ppm corresponding to a polyketide structure with methyl, unsaturated hydroxyl units, respectively. On the other hand, the NMR spectrum of sample 1 showed peaks with smaller intensity at 1.0-1.5 ppm and 3.0-3.5 ppm which might be due to the fact that the sample was diluted (not concentrated via freeze drying). Surprisingly, samples 2 and 4 which were the insoluble fractions of the CFCS and CCS, showed no presence of any polyketide structure but merely a peak at 2.5 ppm corresponding to the solvent DMSO-*d6* which verifies the hypothesized precipitation reaction produced by the antimicrobial producing strain previously mentioned in Section 3.3.4.

A modular organization of the secreted antimicrobial compound(s) was created from the imposed spectra of sample1 (soluble fraction of CFCS) and sample 3 (soluble fraction of CCS) using the (Mnova 10.0.1) software (Figure 5.8). Results showed that sample 3 contains a secondary metabolite similar to that of erythromycin which is a polyketide produced by type I PKSs. This antimicrobial compound is synthesized via three enzymes DEBS1, DEBS2 and DEBS3.

Results also showed the presence of another modular organization but did not reveal the end - product of the biosynthetic pathway which confirms the presence of another secreted antibacterial compound, similar to rapamycin as indicated by the presence of double bonds at the 7.0-9.0 ppm region of the NMR spectrum.

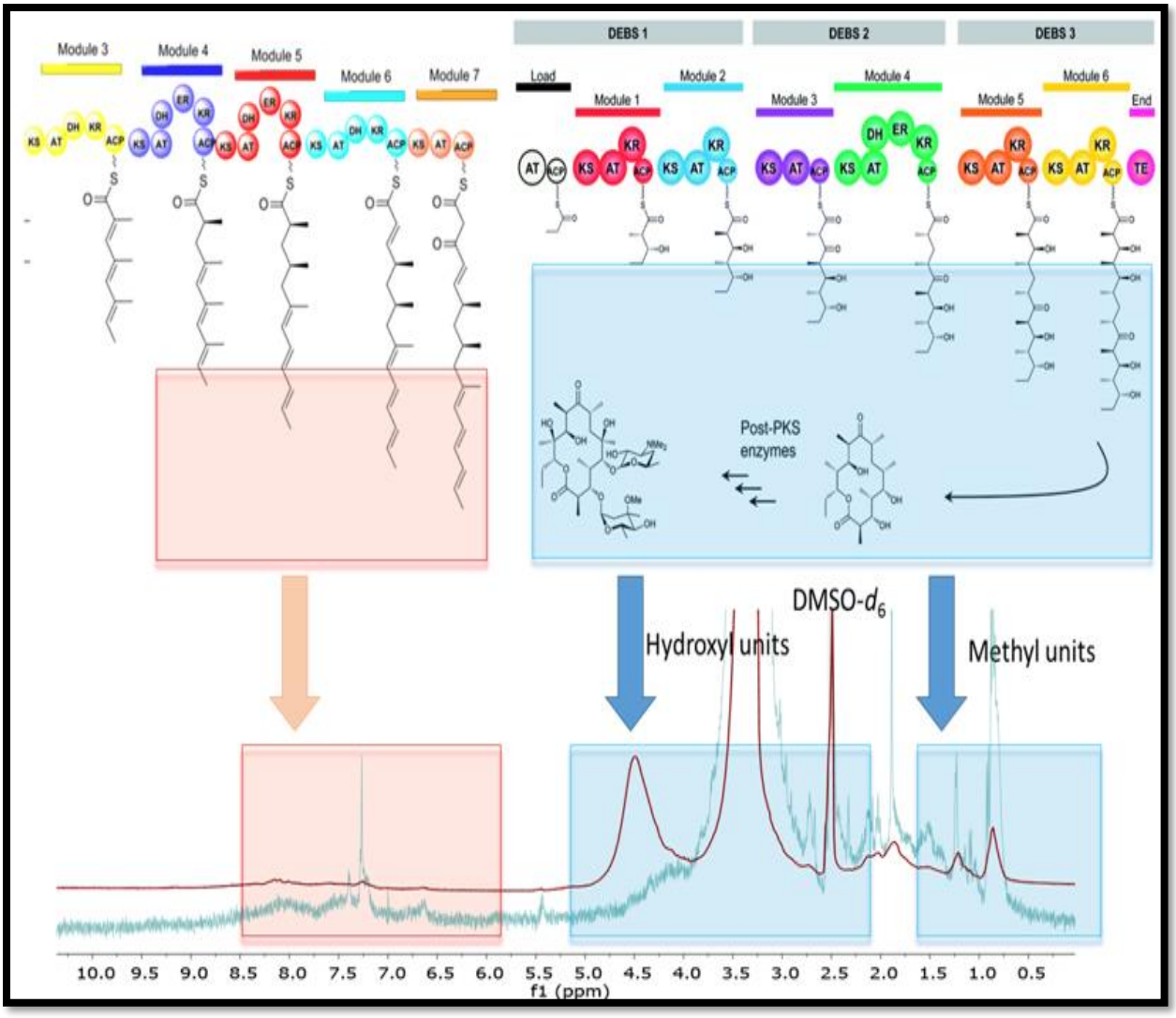

Figure 5.8: NMR analysis of the secreted antimicrobial compound(s) in sample 1 (soluble fraction of the CFCS) and sample 3 (soluble fraction of the CCS). Upper graph; modular organization of the secreted antibacterial compound showing type I PKSs structure resembling erythromycin and incomplete modular organization of a second antibacterial compound similar to rapamycin. Lower graph; superimposed spectra of sample 1 (blue line) and sample 3 (red line) showing a peak at 2.5 ppm corresponding to the DSMO-*d6*, a peak at 0.5-1.5ppm corresponding to methyl units, a peak at ~3-5 ppm corresponding to hydroxyl units and unsaturated moieties at 6-8.5 ppm.

5.3.1 COrrelation SpectroscopY (COSY)

In order to confirm the postulated structure of the secreted antibacterial compounds obtained from the ^{1}H NMR results, a COSY NMR (25 scans) was performed on sample 3 (soluble fraction of CCS).

The COSY spectrum (Figure 5.9) is a representative of an ^{1}H-^{1}H NMR spectrum which runs across a diagonal ridge from the upper right corner to the lower right corner of the plot. Each axis is calibrated based on the ^{1}H chemical shift values (ppm) and can identify unknown compounds via spin–spin couplings of adjacent protons which is manifested as distinctive cross peak patterns (Xi *et al*., 2007). Interpretation of the results was done by extending the vertical and horizontal lines from each spot on the diagonal to its corresponding peak on each axis. It should be noted that the diagonal peaks are merely reference points. The COSY spectrum showed a correlation between the CHOH and alkyl units corresponding to 3.0-4.5 ppm and 1.0-2.5 ppm.

The above results verify those shown in Section 4.4 in that the secreted antimicrobial compound(s) is synthesized by PKSs. Results also confirm the presence of two antimicrobial compounds in the CFCS; one or both responsible for the secreted antibacterial activity.

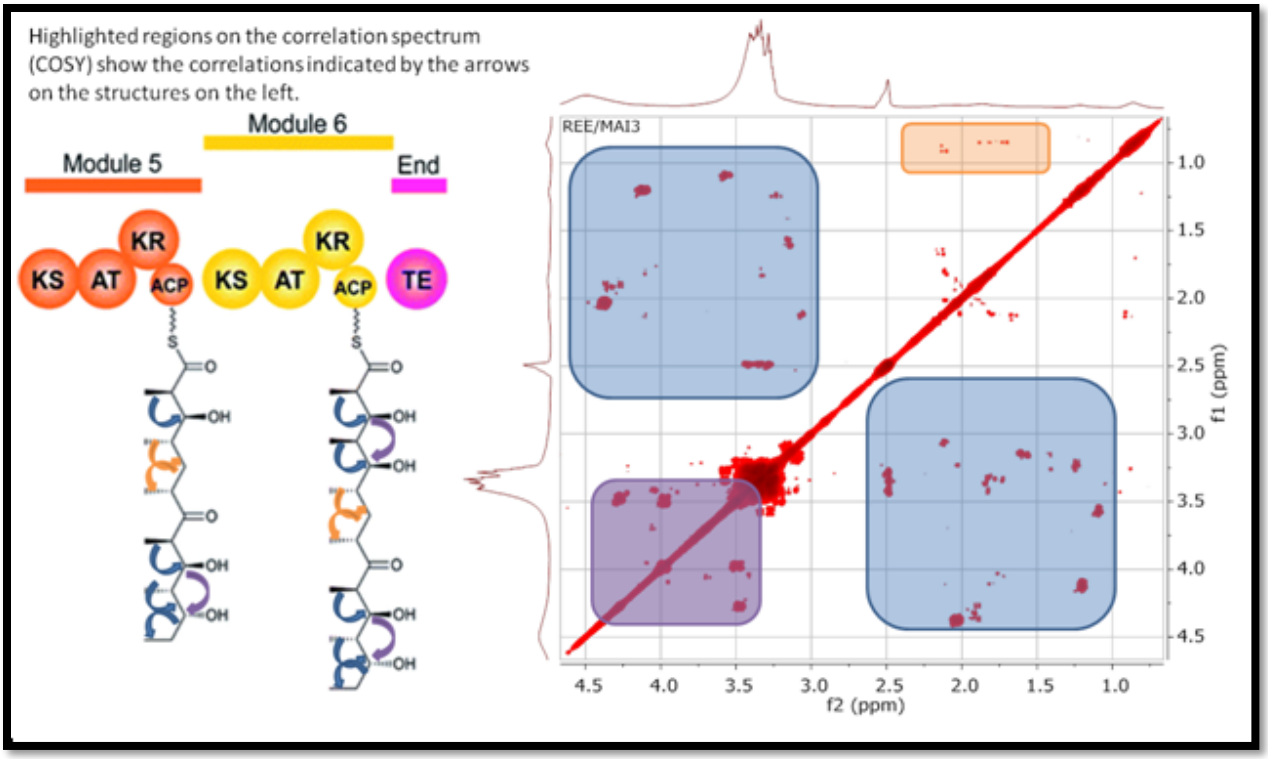

Figure 5.9: Results of COrrelation SpectroscopY COSY of sample 3 (soluble fraction of the CCS) which confirmed a correlation between CHOH moieties with the alkyl units as shown by the arrows that correspond with cross peaks observed in the spectrum highlighted with the same colour as the arrows.

CHAPTER 6: DISCUSSION AND CONCLUSION

6.1 General discussion

The marine environment is an untapped resource of natural metabolites and encompasses an enormous level of rich ecological biodiversity. These compounds exhibit many bioactive properties as natural means of defense against predators (Ibrahim *et al*., 2014; Levy, 2002; Su *et al*., 2016; Vivas *et al*., 2000). Marine derived microbial secondary metabolites are attracting attention due to the rise of antibiotics resistance and the growing demand for finding new antibiotics to combat infectious diseases. These compounds are characterized by antibacterial, anticoagulant, anti-inflammatory, anti-fungal, antihelmintic, antiplatelet, antiprotozoal and antiviral activities as well as acting on the cardiovascular, immune and nervous systems with promising possibilities in marine biochemistry, microbiology and biotechnology as well as in pharmaceutical industry, drug development and research (Malve, 2016; Mayer *et al*., 2010; Kasanah & Hamann, 2004).

Antimicrobial resistance is one of the biggest threats to global public health and efficient continuous collaborations between academics and industrial scientists is essential to ensure the success of marine natural products as novel therapeutic entities (Lehtinen *et al*., 2017; O'Connor, 2015). Strains of the genus *Serratia* inhabit diverse environmental niches with numerous strains considered a reservoir of structurally unique and biologically significant novel secondary metabolites with potent activities. These include; anti-bacterial, anti-fungal, anti-protozoal, anti-malarial and cyto-toxic activities (Bezawada *et al*., 2013; Gulani *et al*., 2012; Bhargavi & Prakasham, 2013). The production of some *Serratia* secondary metabolites is due to the presence of PKSs genes and secretion systems (Adam *et al*., 2016; Bhadra *et al*., 2005; Shang *et al*., 2017). The number of microbial compounds isolated from the marine environment continue to increase each year. Marine bacterial species produce novel bioactive compounds with numerous strains isolated from the surface of marine organisms such as algae. This study investigates the antibacterial activity of an environmental isolate of *S. plymuthica* isolated from the surface of the seaweed *Ascophyllum nodosum* from Aberdour beach, Scotland. The strain produces multiple antibacterial activities. An activity detected on agar plates and a secreted activity detected in the CFCS. This indicates that *S. plymuthica* produces antimicrobial compound(s) that can diffuse into

aqueous environments as well as compound(s) that remain closely bound to the outer cell surface and secreted into solid mediums. This phenotypic property might be a defensive mechanism to protect the antibacterial producing strain in its natural marine habitat due to the fact that antibacterial compound(s) bound to the cells can excrete slowly and continually into the environment preventing colonization by competitors, since a rapid release of antibacterial compound(s) would not provide a competitive advantage due to their immediate elimination by seawater (Hosny *et al*., 2011).

6.2 Preparation of cell-free culture supernatants CFCS

Since the aim of this study is to investigate the secreted antibacterial activity present in the CFCS of *S. plymuthica*, first it was essential to prepare a CFCS with the desired antibacterial activity. This was done by inoculating the antibacterial producing strain in an appropriate broth medium followed by incubation at 25^{o}C for 48h. The bacterial culture was centrifuged, and the culture supernatant was heat-sterilized at 70^{o}C for 10 mins in order to eliminate the presence of any remaining cells and the presence of activity was recorded as average zones of inhibition against the Gram-positive strain (F).

The findings shown in Section 3.2.1 are similar to a study by (Someya & Kataoka, 2000) who reported the presence of two antifungal compounds in the culture supernatant of *Serratia marcescens* Strain B2. The strain was incubated in LB broth at 28°C with shaking for 72h followed by centrifugation (10,000 rpm/5mins) and heat sterilization (2h/60°C) or autoclaving for 20mins. The culture supernatant showed potent antifungal activity against soil-borne fungi such as *Rhizoctonia solani* and *Fusarium*.

Regarding the thermal stability of the secreted antibacterial activity, Stancu, (2016) reported the thermal stability of extracellular secondary metabolites produced by *S. marcescens*. While Bhargavi & Prakasham, (2013) reported the isolation of a macromolecule compound from the novel strain *Serratia marcescens*, RSPB11. This compound showed thermostable properties and tolerated temperatures over 60^{o}C. Also, Zarei *et al*., (2011) isolated a thermo-stable antifungal compound from *Serratia marcescens* B4A. The compound inhibited the growth of plant pathogenic fungi such as *Rhizoctonia solani* and *Alternaria* species. Besides, anti-insecticidal thermostable compounds have been isolated from the culture supernatant of *Serratia* sp. E-15 and *Serratia* sp. RSPB11 (Wencewicz, 2016; Wu *et al*., 2016).

6.3 Optimization of secreated antibacterial production

When studying antimicrobial secondary metabolites, it is very important to determine the most suitable physical factors that enhance their production and resemble their natural environment for future utilization and drug development. Culturing parameters can impact the amount and types of compounds produced by marine microorganisms. These include cultivation temperatures, aeration, incubation time and media composition (Abel *et al*., 1999; Sa´nchez *et al*., 2010 b).

6.3.1 Influence of incubation period on the production of secreted antibacterial activity

This study demonstrated a successful optimization of the laboratory parameters that facilitate the production of the antibacterial activity by *S. plymuthica* by studying the influence of different growth conditions, incubation period, cultivation temperatures and media composition on the production of the secreted activity in the CFCS of the antibacterial producing strain.
The secreted antibacterial activity can be detected in the CFCS after growing the antibacterial producing strain for 24h with highest activity reported btween 48h and 72h (Section 3.2.2) resembling the characteristic trait of secondary metabolites. These results are in accordance with a study by Jafarzade *et al*. (2013) that reported the isolation of an antimicrobial compound from the marine isolate *Serratia* sp. WPRA3 after 72h of incubation. The compound showed potent antibacterial activity against Gram-positive bacteria.

6.3.2 Influence of cultivation temperatures and media composition on the production of secreted antibacterial activity

This study showed that there is a strong link between cultivation temperatures and the production of secreted antibacterial activity in the CFCS of *S. plymuthica*.
Growing the antibacterial producing strain at 16^{o}C resulted in the production of the highest amount of activity detected in the CFCS against the Gram-positive indicator strains in comparison to the other cultivation temperatures as previously mentioned in Section 3.2.3. These findings are compatible with Sa´nchez *et al*. (2010 a) who reported the highest amount of antimicrobial activity in the culture supernatant of *Serratia proteamaculans* 136 when the antibacterial producing strain

was cultivated at temperatures ranging from (4ºC-20ºC) while, the lack of antimicrobial activity when the strain was cultivated at (30ºC–37ºC). Another study by Matilla *et al*. (2016) reported the highest amount of antimicrobial activity produced by *Serratia* A153, when the producer strain was cultivated at 15ºC, slightly present when cultivated at 25ºC but completely absent when cultivated at 30ºC. Also, Jafarzade *et al*. (2013) reported the lack of inhibitory activity of *Serratia* sp. WPRA3 against *S. aureus*, *B. subtilis* and MRSA when the antibacterial producing strain was cultivated at 32ºC.

The manipulation of culture conditions can be used to improve the secretion of secondary metabolites by antibacterial producing strains and the slightest change in growth media composition can change the amount of bioactive compounds induced (Reen *et al*., 2015). Regarding the influence of media composition on the production of secreted antibacterial activity, results showed media-dependent effects (Section 3.2.3). The highest amount of antibacterial activity detected against the Gram-positive strains was from the CFCS obtained from MRS broth. These findings are similar to a study by Petersen & Tisa, (2013) who investigated the secondary metabolites; bacteriocins and serraticin produced by *S. proteamaculans* 136. These bioactive compounds inhibited the growth of Gram-positive and Gram-negative bacteria. The highest amount of antibacterial activity was reported when the antimicrobial producing strain was cultivated in rich medium. A study by Aisyah *et al*. (2016) reported the importance of peptone in growth media in triggering the production of antimicrobial activity in some strains of *Serratia*. Another study by Malheiros *et al*. (2015) reported the significance of MRS broth in enhancing the production of antimicrobial compounds by Gram-negative bacteria.

6.3.3 Influence of different growth environments on the production of secreted antibacterial activity

The production of secreted antibacterial activity in the CFCS of *S. plymuthica* is significantly affected by cultivation parameters such as aeration and culture vessels (Section 3.2.4).

Growing the antimicrobial producing strain in 96 well- plates and 50 ml centrifuge tubes produced inconsistent results which could be due to the lack of proper aeration. Another possible reason could be due to unequal distribution of heat during the heat sterilization step; the 96 well-plate was placed in a hot water bath at (70ºC/30mins). Also, the relatively small volume of broth medium

(~300 µl) in each well left no room for the centrifugation step which is an essential in eliminating *S. plymuthica* cells for the proper detection of the secreted antibacterial activity in the CFCS. On the other hand, growing the antibacterial producing strain in 250 ml flasks created a good aerated environment for the production of the secreted antibacterial activity. Studies on the antimicrobial activity of *Serratia*, emphasized the importance of sufficient aeration on the maximum production of antimicrobial compounds and many used shake-flask fermentations 250 ml flasks to investigate the production of antimicrobial activity (Pansuriya & Singhal, 2011; Houdt *et al*., 2014; Martinez *et al*., 2015). The marine isolate *Serratia* sp. WPRA3 showed the highest amount of antibacterial activity against the Gram-positive strains *S. aureus* and *B. subtilis* after the antimicrobial producing strain was cultivated for 72h at 200 rev/min (Jafarzade *et al*., 2013; Shi *et al*., 2014).

6.4 Characterization of secreted antibacterial activity

In order to get a better understanding of the secreted antibacterial activity, the effect of different physio-chemical conditions on the stability of the activity was investigated.

6.4.1 Effect of storage on the stability of secreted antibacterial activity

Several studies reported the isolation of cold-active antimicrobial secondary metabolites from psychrotrophic bacteria such as; *S. proteamaculans* 136 and *S. liquefaciens* (Hemala *et al*., 2014; Machado *et al*., 2016; Sánchez *et al*., 2010).

The effect of storage at 4^{o}C on the stability of the secreted antibacterial activity detected in the CFCS was studied by recording the presence of activity against the Gram-positive strain (F). Results showed that the activity decreases overtime during storage as previously shown in Table 3.7 which might indicate that secreted antibacterial compound(s) of *S. plymuthica* are novel and different to other reported *Serratia* antibacterial compounds.

6.4.2 Effect of Proteinase K, TWEEN® 20 and the pH on the stability of secreted antibacterial activity

Several studies reported the isolation of novel *Serratia* antimicrobial secondary metabolites that are insensitive to proteinase K treatment such as the protein antifungal compound produced by *Serratia marcescens* DT. This compound maintained its inhibitory activity towards *Rhizoctonia solani* and *Fusarium oxysporum* even after treatment with proteinase K (0.5–4 μg/mL) (Do *et al*., 2017). Also, *Serratia marcescens* Mm3 produces a heat-stable bacteriocin antibacterial compound that is insensitive to organic solvents and proteinase K (Uğras *et al*., 2014).

This study showed that the secreted antibacterial activity was insensitive to proteinase K indicating that the antibacterial compound(s) is unlikely to be a protein. Opposite results were reported by Valdez *et al*. (2008) on the loss of antimicrobial activity of the protein toxins of the biocontrol agent *Serratia entomophila* Mor4.1 toward beetle larvae when treated with proteinase K.

Results also showed that the secreted antibacterial activity remained active after incubation with the detergent Tween 20 (Section 3.3.2). Similar results were reported by Li *et al*. (2011) on the

secondary metabolites isolated from the culture supernatant of *Serratia* sp. SYBC H. The compounds showed hydrophilic characteristics and their activity was not inhibited by non-ionic detergents like Tween. Regarding the influence of pH on the stability of the secreted antibacterial activity, the highest recorded activity was measured at (pH 7.3±0.2) before changing the pH of the CFCS. The activity was slightly affected but not inhibited by changes to the pH. These findings are similar to numerous studies regarding the pH stability of *Serratia* secondary metabolites such as the antifungal and antimicrobial compounds produced by *Serratia marcescens* B4A and *S. proteamaculans* (Zarei *et al*., 2011; Hemala *et al*., 2014; Solé *et al*., 2000).

6.4.3 Effect of Tris buffer on the stability of secreted antibacterial activity

The addition of buffers maintains a relatively stable pH during the extraction and purification of bacterial secondary metabolites including antimicrobial agents (Thakur *et al*., 2016; Ahmad & Wu, 2012; Salarizadeh *et al*., 2014; Yang *et al*., 2014). Results showed that the secreted antibacterial activity was affected by the addition of Tris-buffer (Section 3.3.4) making it an unsuitable choice to be used as a preservative. Further studies are needed to find the optimum medium for the long-term storage of the secreted activity.

The results of the NMR spectrometry (Section 5.3) verified the hypothesized precipitation reaction produced by *S. plymuthica* observed in Section 3.3.4, indicating that the secretion of specific *Serratia* enzymes by the antibacterial-producing strain metabolize organic acids and glycerol, causing a precipitation reaction; organic acids and glycerol are components of the Tris-buffer and NB broth (Newsome *et al*., 2015; Hejazi & Falkiner, 1997; Ugwu & Apte, 2004).

6.4.4 Molecular weight estimation of the secreted antibacterial compound(s)

Serratia marcescens Strain B2 secretes two antifungal compounds in the culture supernatant. A thermo-sensitive high-molecular weight compound and a heat-stable low-molecular weight compound (Someya & Kataoka, 2000). Estimation of the molecular weight of the secreted antibacterial compound(s) present in the CFCS was done using centrifugal filter units which are commonly utilized for the isolation and concentration of bacterial bioactive compounds (Erb *et al*. 2010; Paiva *et al*., 2013). The present study indicates the possible presence of two secreted

antibacterial compounds in the CFCS; a compound with a molecular weight ≥10,000 Daltons and a compound with a molecular weight >3000 and <10,000 Daltons. It is also possible that there is only one antimicrobial compound and that some of it forms larger aggregates. However, results obtained via NMR analysis in Section 5.3. contradicts the latter possibility and confirm the presence of two antimicrobial compounds in the CFCS.

Polyketides vary in molecular weight according to functional group modifications (Civjan, 2012). Another approach in determining the size of the secreted antimicrobial compounds would be by performing gel-filtration chromatography on the CFCS in order to separate and purify the secreted antibacterial compounds according to their molecular size (Ó'Fágáin *et al*., 2011; Hong et *al*., 2012; Carbonell *et al*., 2003; Nawani & Kapadnis, 2001).

6.4.5 The bactericidal activity of the secreted antibacterial compound(s)

Studies reported the production of secreted antimicrobial compounds in the culture supernatant of *S. marcescens* and *S. nematodiphila* with potent bactericidal activity against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis* and *K. planticola* (Malarkodi *et al*., 2013; Akilandeswari *et al*., 2014 Arivizhivendhan *et al*., 2015). Also, several strains of *Serratia* produce heat-stable antibacterial compounds that are insensitive to proteinase K and active against various Gram-positive bacteria such as; *B. cereus*, *B. subtilis* and *S. aureus* (Matilla *et al*., 2016; Gerc *et al*., 2012). The novel strain, *Serratia* sp KC149511 isolated from algae possess potent antibacterial and antifungal activity (Karthick *et al*., 2015). Some strains of *S. plymuthica* produce lytic activity as a protective mechanism. These strains produce antimicrobial compounds with broad spectrum antibacterial and antifungal properties. An anti-fungal compound was detected in the culture supernatant of a novel strain of *S. plymuthica* when grown in liquid medium for 72h. The compound was thermostable and active against *Cladosporium* and *Fusarium* (Jankiewicz & Brzezinska, 2015; Frankowski *et al*., 2011).

Results previously mentioned in Section 3.3.7 showed that the secreted antibacterial compound(s) present in the CFCS has species-specific properties, targeting Gram-positive bacteria in particular those belonging to *Bacillaceae* These results are similar to Dusane *et al*. (2011) who reported the antimicrobial activity of the cell-free culture supernatant of the marine bacterium *Serratia marcescens* isolated from the coral *Symphyllia*. The CFCS inhibited the growth of the Gram-

positive pathogens; *C. albicans* and *Bacillus pumilus*. Gram-positive bacteria are known for their susceptibility to antibiotics and antimicrobials due to the lack of the outer lipopolysaccharide membrane and porins proteins (Darabpour *et al*., 2010).

6.5 Transposon mutagenesis

Molecular biology methods are important tools for the identification of bacterial genes responsible for the production of secondary metabolites (Banik & Brady, 2010; Cragg & Newman, 2013). Transposons are mobile genetic elements; segments of DNA that move from one site to another on the same genome or different DNA molecules; leading to the disruption of the region of the genome where the transposon has been inserted and hence the name (gene knock-out) (Lewenza *et al*., 2005; Judson & Mekalanos, 2000; Bennett, 2008). This creates chromosomal mutations such as; DNA insertions, inversions, deletions and chromosomal fusions (Lamberg, 2002; Goryshin *et al*., 2000; Ferrieres *et al*., 2010; Veeranagouda *et al*., 2012; Reznikoff, 1993).

Random transposon mutagenesis inactivates gene function by creating stable single insertions in the bacterial host genome as a result of high frequency of transposition and low sequence insertion specificity. Transposons are valuable in constructing mutant libraries of strains containing a single randomly located transposon based on an observed phenotype whose genotype was disrupted as a result of transposon insertion into the genome (Winson *et al*., 1998; Opijnen & Camilli, 2013; Buchan *et al*., 2008; Reznikoff *et al*., 1999).

In this study, transposon mutagenesis was performed via conjugation between the environmental strain *S. plymuthica* and an *E. coli* strain carrying plasmid pRL27 with the aim of identifying gene(s) responsible for the production of the secreted antibacterial activity detected in the CFCS. Plasmid pRL27 is a successful transposon delivery vector used with a variety of bacterial species including; *Xanthobacter autotrophicus*, *Pseudomonas*, *E. coli* and *Citrobacter rodentium* (Lyell et *al*., 2008; Choi *et al*., 2009; Martínez *et al*., 2014).

pRL27 is a suicide plasmid and mobilized via the control of the origin of transfer (*oriT*) from plasmid RP4 (Lorenzo *et al*., 1990; Larsen *et al*., 2002). During conjugation, the transfer of genetic material requires a direct contact between the donor and the recipient cell. A pilus pulls the two cells together and a bridge is formed; a single strand of plasmid DNA passes into the recipient cell then becomes double stranded (Freeman, 2000; Bruckbauer *et al*., 2015; Braam *et al*., 1999).

The plasmid also carries a mini-Tn*5* transposon that encodes resistance to kanamycin Km^R and a transposase gene (*tnp*) which is controlled by the *tetA* promoter (*tetAp*) from plasmid RP4. The *tetAp::tnp* gene is located outside the transposon so after transposition the *tnp* gene is lost, leading to stable transposon insertions (Naumann & Reznikoff, 2002; Reznikoff *et al*., 2003; Adams *et al*.,

2006). Hence, transposon insertions obtained in this study do not contain the *tnp* gene and cannot induce further transposase-mediated events. They are stable even in the absence of antibiotic selection (Hayes, 2003; Hoffman *et al*., 2000). The Tn*5* also contains an origin of replication from plasmid R6K (*ori*R6K) that allows cloning of transposon insertion sites and replication is dependent on the π protein encoded by the *pir* gene. Since *S. plymuthica* does not contain the π protein, Km^R transconjugants can only be obtained if the transposon inserts into the recipient genome. The transposon insertion with adjacent genomic DNA can be maintained as a plasmid in a host with the *pir* gene; allowing cloning and sequencing of transposon insertion sites (Zhang *et al*., 2000; Ferrieres *et al*., 2010; Ravindran, 2012; Herrero *et al*., 1990).

6.5.1 Screening for mutants deficient in the secreted antibacterial activity

While primary bacterial metabolic processes can be found in many bacterial species. Genes responsible for the biosynthesis of secondary metabolites are very specialized in which certain biochemical pathways are found in restricted bacterial species (Selenko &Wagner, 2007; Strobel *et al*., 1999).

One might argue that secreted antibacterial activity of the wild type *S. plymuthica* detected in the CFCS is related to the antibacterial activity detected on agar plates. But if this was the case, then transposon mutagenesis would only produce one type of mutant that is deficient in both activities; (the one identified on agar plates and the one identified in liquid medium). However, this study reports the isolation of two Km^R transposon mutants; M74 and M426 with phenotypic bioactivity opposite to those mutants previously isolated by AlThubiani, (2013). Both Km^R transposon mutants were deficient in the secreted antibacterial activity but still possessed the antibacterial activity detected on agar plates.

6.5.2 Identification of transposon insertion sites

Identification of transposon insertion sites was achieved by cloning of transposon insertions. This was done by isolating genomic DNA of the Km^R transposon-induced mutants M74 and M427, followed by enzymatic digestion of the DNA and ligation with T4 DNA ligase. The digested genomic DNA of Km^R mutants was introduced into an *E. coli* strain that contains the *pir* gene so that circularized DNA fragments containing the transposon would replicate as *pir*-dependent plasmids. There are various reasons that might have affected the success of transformation in this study when using home-made *E. coli* S17-1 λpir (Section 4.4). The occurrence of few or no transformant cells indicate that the competent cells are not sufficiently competent (Zhou *et al*., 1998). Another possible reason of transformation failure when using *E. coli* S17-1 λpir as vectors is that, these strains contain the phage Mu genome that can mobilize itself into the recipient cell and in turn randomly mutate the recipient genome or even the carried plasmid (Dominguez & O'Sullivan, 2013; Tolonen *et al*., 2006; Strand *et al*., 2014). Regarding the KmR mutant M74, failure to obtain DNA sequencing results from recombinant plasmids could be due to numerous reasons; which might have occurred during electroporation that sometimes can cause DNA degradation especially when large DNA fragments are cloned in high copy number causing illegitimate recombination (Dennis & Zylstra ,1998; Dominguez & O'Sullivan, 2013). Other reasons could be due to insufficient or poor DNA quality, presence of contamination in sequencing solutions, degraded primers and low concentration of plasmid DNA (Goryshin & Reznikoff, 1998; Braam & Reznikoff, 1998).

6.5.2.1 Microbial biosynthetic pathways

In recent years, the rate of discovery of bioactive compounds has decreased and improved screening systems are required to overcome this problem (Komaki *et al*., 2009; Sakurai *et al*., 1996; Akele *et al*., 2015). The biosynthesis of secondary metabolites remains poorly understood across a wide variety of antimicrobial producing species. Also, the number of microbial biosynthetic genes greatly exceeds the number of known compounds produced by these microorganisms emphasizing the term "silent/cryptic" genes. Research revealed the presence of cryptic genes in many anti-microbial producing microorganisms that even with advanced culturing

technologies; many remain an untapped source of novel metabolites. This might be due to the fact that under laboratory cultivation conditions, these compounds are produced in small amounts that cannot easily be detected. Also, biosynthetic genes might not be expressed because their activation relies on specific environmental triggers that are missing in laboratory settings (Reen *et al*., 2015; Komaki *et al*., 2009; Su *et al*., 2016). This study reports that the secreted antibacterial activity detected in the CFCS of the wild type *S. plymuthica* is synthesized by PKSs that are distinct from other known *Serratia* PKSs. Results of this study are consistent with both suggested possibilities of the hypothesis previously mentioned in Section 4.4 in which the antibacterial producing strain *S. plymuthica* might possess separate biochemical pathways leading to two distinct activities or have a branched biochemical pathway. However, a knowledge gap still exists between the hypothesis and the involvement of PKSs in the production of the observed antibacterial activities. Further investigation is absolutely required to realize the full potential of this novel isolate and to unveil if the operons responsible for the two activities are in separate genomic locations and regulated in different manners which would finally prove that the strain possesses two separate biochemical pathways leading to the two distinct activities. Microbial genomics is a rapidly developing science to discover novel secondary metabolites for drug discovery. Performing a complete genome sequence of *S. plymuthica* would unlock many unanswered questions and reveal valuable insights regarding genes functions and regulations (Chung *et al*., 2013; Li *et al*., 2015; Bachmann, 2014).

6.6 Purification of the secreted antibacterial compound(s) by organic solvent extraction

In microbiological samples, antimicrobial compounds usually represent <1% of the original mixture and studying these compounds begins by extraction from crude samples followed by purification and identification (Peláez, 2006; Penesyan, 2010).

In this study, purification of the secreted antibacterial compound(s) was preceded by concentrating the CFCS via freeze drying. Freeze-drying can be used to effectively dry microbiological samples including those containing antimicrobial compounds through dehydration and sublimation of ice without affecting the integrity of the compounds of interest. This method also prevents microbial growth and hence might improve the storage stability of some bioactive compounds (Shofian *et al*., 2011; Mphahlele *et al*., 2016; Perry, 1998).

Regarding the solubility of secreted antibacterial compound(s) in methanol (Section 5.2). Several studies reported the solubility of *Serratia* antibacterial compounds in water and organic solvents (Li *et al*., 2011; UĞRAŞ *et al.,* 2014; Pore *et al*., 2016) including the secreted compounds in *Serratia* sp. SYBC H culture supernatant and the thermostable secondary metabolites of *Serratia marcescens* (Kaira *et al*., 2015; Hejazi & Falkiner, 1997).

The secreted antibacterial compound(s) detected in the CFCS of the wild type *Serratia plymuthica* resembles the peptide antibiotic marcescin isolated from the culture supernatant of *S. marcescens* (Fuller & Horton, 1950). Both compounds are thermostable, bactericidal and inhibit the growth of Gram-positive bacteria and their production is influenced by aeration and temperatures below 25^{o}C. Both compounds are soluble in water and organic solvents and their antimicrobial activity fades after few days even when stored at low temperatures (0-4^{o}C). Also, both compounds tend to precipitate when solvent is added forming a voluminous jelly.

6.7 NMR and COSY NMR spectroscopy

NMR spectroscopy allows structural investigations at the atomic level and is efficient in analyzing complex biological mixtures such as culture supernatants of microbial cultures and in the detection and identification of low molecular-weight metabolites even at low concentrations (AlMajidi, 2014; Pegos *et al*., 2014; Gerwick & Fenner, 2013; Kwon *et al*., 2014; Gouda *et al*., 2010).

NMR is a rapid, high-throughput analysis technique, accurate and non-destructive with minimal sample preparation; no necessity for purification of compounds from original mixtures (Brennan, 2014; Fan & Lane, 2016; Bharti & Roy, 2012; Singh & Pheko, 2008). Likewise, COSY NMR is a sensitive, rapid, automated analytical technique used for the detection of coupling interactions of unknown natural secondary metabolites in order to identify their molecular structure. The COSY spectrum encompasses an NMR spectrum that runs across a diagonal ridge from the upper right corner to the lower right corner of the plot. Each axis is calibrated based on the chemical shift values (ppm). In COSY NMR, the identification of unknown compounds can be achieved via their spin–spin couplings manifested by distinctive cross peak patterns (Kempgens & Pinchuck, 2016; Winden *et al*., 2001; Xi *et al*., 2007).

In this study, results of NMR and COSY NMR spectroscopy showed that the CFCS contains a secondary metabolite resembling the antibacterial antibiotic erythromycin produced by 6-deoxyerythronolide B synthases (DEBS) which are large multifunctional enzymes belonging to type I PKSs (Sanchez *et al*., 2010 b; Pereda *et al*.,1998; Mcdaniel *et al*.,1999). Results also showed the presence of another antibacterial compound in the CFCS with similarities to rapamycin indicated by the presence of double bonds at the 7-9 ppm region of the NMR spectrum (Section 5.3). Polyketides produced by type I PKSs such as erythromycin and rapamycin are used as anti-bacterial and immune-suppressant compounds (Cane, 2010). Both erythromycin and rapamycin are macrolide antibiotics and protein synthesis inhibitors. Erythromycin can be used to treat respiratory tract infections such as; bronchitis, pneumonia. Rapamycin is an immunosuppressant prevents organ transplant rejection (Bedford *et al*., 1996; Hill *et al*.,2017; Li *et al*., 2014)

PKSs are responsible for the biosynthesis of polyketides which are a large and diverse class of natural products created via the condensation and modification of carboxylic acids and involve different enzymatic functions (Crosby & Crump, 2012; Tyc *et al*., 2017; Shelest *et al*., 2015). For many years now, PKSs of bacterial origins have been shown to be responsible for the production

of various novel secondary metabolites (Zhang *et al*., 2009). There have been numerous studies regarding marine derived polyketides, but much remains to be done in characterizing marine microbial PKSs and understanding their associated biological functions (O'Connor, 2015; Penesyan, 2010; Staunton & Weissman, 2001). PKSs are arranged in clusters in bacterial genomes and are responsible for the biosynthesis of numerous diverse secondary metabolites many of which are used as anti-cancers and antibiotics (Donadio *et al*., 2007; Masschelein *et al*., 2015a).

6.8 Conclusion and future work:

6.8.1 Conclusion:

Serratia species synthesize a diverse array of secondary metabolites with unique biological properties and represent a promising valuable source for research and understanding the biochemical nature of these compounds is the key for future development of new drugs and antibiotics. Results of this study concluded the following:

1- To our knowledge, this is the first study regarding an environmental isolate of *S. plymuthica* capable of producing two antibacterial activities both encoding PKSs.

2-Different cultivation parameters such as; culture medium, aeration and temperature have a clear impact on the production of the secreted antibacterial activity detected in the CFCS which might help in future large-scale production of the antimicrobial compounds.

3- The secreted antibacterial activity was thermo-stable and remained active even after sterilization (121°C/15mins).

4- The secreted antibacterial activity was insensitive to methanol, Proteinase K, Tween and pH and therefore has potential in industrial and environmental biotechnology, food industry, and bioremediation.

5- The secreted antibacterial activity was bactericidal and a promising antibacterial agent against some Gram-positive bacteria.

6- The secreted antibacterial activity fades after few days even when stored at (4^{o}C) and precipitates in the presences of solvents.

7- NMR and COSY NMR spectroscopy showed that the CFCS contains two secondary metabolites resembling the antibiotics erythromycin and rapamycin produced by DEBS.

6.8.2 Future work:

Modern medicine relies heavily on antibiotics to combat bacterial infections. Antibiotic resistance is a serious threat to humanity and a challenge to modern medical practices. Many countries and international agencies around the world have implemented numerous policies to hinder the spread of antibiotic resistance in clinical practices and in the community. The development of new antimicrobial compounds is urgently needed before the current antibiotics used in clinical settings become compromised by antibiotic resistance. The range of *Serratia* species is vast and produces an enormous group of secondary metabolites with different unique bioactive compounds and provide an excellent basis for investigation for future drug discovery and the expansion of the pharmaceutical pipeline. Many bacterial species encompass PKS genes however, many are not expressed under normal laboratory conditions. Despite the extensive research in the field of microbial secondary metabolites, knowledge of the structure and function of many polyketides still remains a mystery. Further studies are needed for the molecular characterization and purification of the secreted antibacterial compounds present in the CFCS as well as the identification of other secondary metabolites that might be produced by this novel strain of *S. plymuthica* to unveil any underlying biosynthetic pathways which might reveal the presence of new compounds with future importance.

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